ANTIMICROBIAL EFFECTORS ACT COOPERATIVELY TO STRESS *SALMONELLA* IN THE MACROPHAGE PHAGOSOME

BY

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DISSERTATION

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Abstract

Salmonella Typhimurium survives and replicates in host macrophages. The bacterium produces two Cu-Zn periplasmic superoxide dismutases, SodCI and SodCII. Although both enzymes are expressed during infection, only SodCI contributes to virulence in the mouse by combating phagocytic superoxide. We have shown that the differential contribution to virulence is due to inherent differences in the SodCI and SodCII proteins that are apparently independent of enzymatic activity. Our goal is to understand what features of these proteins are required for virulence in the animal. SodCII is a monomer, is protease sensitive, and is released by osmotic shock, like other known periplasmic proteins. In contrast, SodCI forms dimers, is protease resistant, and is retained within the periplasm after osmotic shock, a phenomenon that we term tethering. We are further characterizing SodCI tethering within the periplasm and its importance for *Salmonella* virulence. We hypothesize that during infection, cationic antimicrobial peptides (CAMPs) from the macrophage transiently disrupt the outer membrane. SodCII is released from the periplasm and degraded. SodCI is both tethered within the periplasm and is protease resistant. This hypothesis provides a rationale for why tethering is important for virulence. We have found that osmotic shock and polymyxin B treatment are analogous. SodCII is preferentially released by polymyxin B, while SodCI is retained. Likewise, mouse macrophage Cathelicidin-related antimicrobial peptide (CRAMP) treatment leads to SodCII release, while SodCI is tethered within the periplasm. We tested if SodCII, protected from release by antimicrobial peptides, could contribute to virulence *in vivo*. A *Salmonella pmrA*^{C} mutant is resistant to CAMPs. We show that SodCII enhances

virulence in a *pmrA*^{C} background. These results suggest that SodC tethering within periplasm is important for *Salmonella* virulence.

We are also addressing how SodCI is tethered within the periplasmic space. It was previously shown that SodCI was not found in the membrane fraction and is tethered even when it is over expressed. We also know that this is a non-covalent interaction. Therefore, we hypothesize that it binds to something abundant in periplasm. We can show that SodCI binds to purified peptidoglycan (PGN), whereas PhoA and monomeric SodCI, both of which were released by osmotic shock, do not bind. We also show SodCI does not bind to chitin, staphylococcus PGN but binds bacillus PGN which has same structure of *Salmonella* PGN. This result suggests that SodCI is tethered to peptidoglycan during osmotic shock or antimicrobial peptide treatment.

Lastly, we investigated the relationship between the macrophage host factors and *Salmonella* periplasmic stress. *Salmonella* cope with various antimicrobial substances in macrophages, such as cationic antimicrobial peptides (CAMPs), proteases, and reactive oxygen species, by encoding various virulence factors such as the PmrAB regulon that modifies LPS, the protease inhibitor Ecotin, and the periplasmic superoxide dismutase SodCI. The phagocytic antimicrobial substances presumably initiate damage to the bacterium in the outer membrane or periplasmic space. The Sigma E and the Cpx regulons of *Salmonella* sense and respond to stress in the outer membrane and periplasm, respectively. It is known that these periplasmic stress response regulators are induced and important during the infection, but the nature of the host factors that induce these regulators has not been elucidated *in vivo*. To answer this question and gain insight into

the mechanisms by which phagocytes kill bacteria, we constructed RpoE- and Cpx-regulated *lacZ* fusions to measure the level of stress during infection in the mouse model. To test the effect of cationic antimicrobial peptides (CAMPs) during infection, *htrA-lacZ* (Cpx-dependent) and *rpoE-lacZ* (RpoE-dependent) expression was compared in wild type and *pmrA* mutant backgrounds. The PmrA regulon is required to protect against CAMPs. The *htrA* fusion was specifically induced in the *pmrA* mutant during infection, whereas *rpoE* was not. Interestingly, in vitro, CRAMP (presumed to be the principal antimicrobial peptide and mouse macrophages) and the polymyxin derivative, polymyxin nonapeptide (PMNP), induce both *htrA* and *rpoE*, whereas cecropin induces *htrA* but not *rpoE*. These results suggest that different antimicrobial peptides induce you induce different types of damage to the cell. Extrapolating this result to our in vivo data suggests that the damage occurred in the phagosome is analogous to that used by the secretary in vitro rather than CRAMP.

The effect of superoxide on the *Salmonella* periplasm in the phagosome was also tested by monitoring the expression of these genes in a *sodCI* deletion background. Our results show that *rpoE* expression was induced but *htrA* was not. Neither hydrogen peroxide nor chemically generated superoxide induced *htrA* or *rpoE* in vitro. The periplasmic stress seen in the *sodCI* deletion may be the indirect effect of reactive oxygen species. Lastly, the effect of macrophage proteases was tested *in vivo* by removing the periplasmic serine protease inhibitor Ecotin. Only *rpoE* was induced consistently during infection with the *eco* strain. In the future, the effects of proteases on periplasmic stress will be tested *in vitro*.

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Chapter 1: Introduction

Approximately 1.4 million cases of non-typhoidal *Salmonella* infection are estimated annually in United States (Mead *et al.*, 1999;Voetsch *et al.*, 2004). In humans, *Salmonella enterica* is ingested orally and causes diseases ranging from self-limiting gastroenteritis to systemic enteric fever. In some cases, the infection can result in a chronic asymptomatic carriage. *Salmonella enterica* are classified into 6 subspecies and over 2000 serovars defined by LPS (O) and flagellar (H) antigens (Popoff, 2001). Even though *Salmonella enterica* are genetically very similar, epidemiology and virulence traits vary enormously. Most serotypes cause gastroenteritis in humans while specific serovars such as *Salmonella enterica* serovar Typhi or Paratyphi cause enteric fever (Popoff, 2001;Shere *et al.*, 1998;Miller and Pegus, 2000). A few non-typhoid serovars such as *Salmonella enterica* serovar Choleraesuis or Dublin are more likely to cause bacteremia than diarrhea (Blaser and Feldman, 1981). Some *Salmonella* are fully host adapted like serovar Typhi which only infects humans. Some of them are partially host adapted. For instance serovar Choleraesuis is associated with swine (Ferris and D.A.Miller, 1990), whereas serovar Dublin primarily infects cattle (Bulgin, 1983;Robinson *et al.*, 1984).

Salmonella enterica serovar Typhimurium normally causes a self-limiting gastroenteritis in humans, but it causes a typhoid-like systemic infection in mice, which is similar to Typhoid fever caused by serovar Typhi in humans (Carter and Collins, 1974). Therefore, the mouse model is an excellent tool to study systemic *Salmonella* infections. My thesis

is focused on the virulence of serovar Typhimurium in the mouse model; I hereafter use "*Salmonella*" to refer to *Salmonella enterica* serovar Typhimurium.

1.1 Overview of *Salmonella* Infection

Salmonella are orally ingested in contaminated food or water. There are various defense mechanisms in the host that act against pathogens such as *Salmonella*, but *Salmonella* is also equipped with systems to survive in the harsh host environment. The first barrier to *Salmonella* is the acidic environment of the stomach. After passing through the stomach, *Salmonella* colonize the small intestine. *Salmonella* can gain access to underlying lymph tissue through phagocytic M cell (Jones *et al.*, 1994) or non-phagocytic villous epithelial cells using the *Salmonella* Pathogenicity Island 1 (SPI1) encoded Type Three Secretion System (TTSS). The bacteria preferentially target and invade the most distal Peyer's Patch of the small intestine (Carter and Collins, 1974). After invasion, *Salmonella* is disseminated to various target organs such as liver, spleen and bone marrow where the bacteria replicate within macrophages (Richter-Dahlfors *et al.*, 1997). *Salmonella* is able to grow in virtually all tissues of the body. The infected host dies as a result of septic shock and organ failure.

1.2 *Salmonella* Survival and Replication in Macrophages

Salmonella systemic infection requires survival and replication in macrophages (Oh *et al.*, 1996;Richter-Dahlfors *et al.*, 1997), and *Salmonella* mutants that cannot replicate in macrophages are attenuated in the mouse model (Fields *et al.*, 1986). Once *Salmonella* enters a macrophage, it forms a vacuolar compartment called the spacious phagosome

(SP) (puche-Aranda *et al.*, 1994). Then the SP shrinks and forms a membrane around one or more bacteria which is called the *Salmonella* containing vacuole (SCV). Although many genes are required for *Salmonella* survival, the T3SS encoded on *Salmonella* Pathogencitiy Island 2 (SPI2) plays a major role.

Macrophage lysosomes contain a variety of antimicrobial properties and substances, which include acidic pH, reactive oxygen species (ROS), antimicrobial peptides, proteases and lysozyme. It is controversial whether lysosomes fuse with the SCV. Oh *et al.* and others showed that *Salmonella* can survive in phagolysosomes. It is also clearly shown that late endosomal/lysosomal contents are directly transferred to the SCV in a process very similar to classic phagosome-lysosome fusion, including a prerequisite acidification (Buchmeier and Heffron, 1991;Drecktrah *et al.*, 2007;Oh *et al.*, 1996). On the other hand, Buchmeier *et al* also suggested that *Salmonella* inhibits phagosome-lysozome fusion (Buchmeier and Heffron, 1991). Vazquez-Torres and coworkers showed that the SPI-2 T3SS contributes to *Salmonella* survival by blocking delivery of the NADPH oxidase, which is responsible for production of ROS (Oh *et al.*, 1996;Vazquez-Torres *et al.*, 2000b), to the SCV. Thus, SPI2 apparently inhibits phagolysomal fusion. But this inhibition is not complete and other virulence factors such as members of the PhoP regulon and the periplasmic superoxide dismutation SodCI act to protect *Salmonella* from lysosomal antimicrobial substances (Krishnakumar *et al.*, 2004;Miller *et al.*, 1989;De Groote *et al.*, 1997)

1.3 Host Defense by Macrophages

As mentioned early, systemic infection of *Salmonella* requires survival and replication in macrophages. Generally, macrophages have two general pathways to kill phagocytosized bacteria. One is the oxidative pathway, which is mediated by reactive oxygen species and reactive nitrogen species. The other is non-oxidative pathway which includes proteases and antimicrobial peptides. The following describes representative macrophage defensive systems.

1.3.1 Phagosomal killing by oxidative pathway

Reactive oxygen species

The most notable antimicrobial substances in macrophage are reactive oxygen species generated by the NADPH oxidase. When *Salmonella* is phagocytosed into the macrophage, oxygen consumption is dramatically increased and this is termed the respiratory burst. This is due to a large production superoxide by the NADPH oxidase, which is composed of two membrane-bound components, gp91 and p22, and four cytosolic components, p40phox, p47phox, p67phox and RacGTPase. When phagocytes are activated, p47 phox is heavily phosphorylated and the cytosolic components assemble onto membranes containing gp91 and p22. Subsequently, this complex associates with cytochrome b558 to yield the active oxidase (Babior, 1999). The importance of the respiratory burst is exemplified in chronic granulomatous disease (CGD) patients. CGD is a hereditary disorder cause by a mutation in one of the NADPH oxidase subunits. CGD patients experience recurrent fungal and bacterial infection; *Salmonella* is the second

most prevalent cause of bacterial infections in CDG patients (Johnston, Jr., 2001;Winkelstein *et al.*, 2000).

Reactive Nitrogen Species (RNS)

Another important antimicrobial effector is nitric oxide, produced by the inducible nitric oxide (NO) synthase (iNOS) (Sessa, 1994;Espey *et al.*, 2000). The iNOS dependent nitric oxide production is independent of the NADPH oxidase. There is are reported iNOS mutations in humans, but nitric oxide ($NO\cdot$) can inhibit bacterial growth in vitro through the modification of protein thiols, lipids, and DNA (Wink *et al.*, 1991;Radi *et al.*, 1991;Butler and Megson, 2002). In phagocytes, dimeric iNOS catalyzes L-arginine and O_2, using NADPH, to citrulline and $NO\cdot$. $NO\cdot$ further auto-oxidizes to NO_2 or N_2O_3 or becomes peroxynitrite ($ONOO^-$) by reacting with O_2^-. Both N_2O_3 and $ONOO^-$ are capable of modifying nucleotides (Nathan and Shiloh, 2000). Data suggest that iNOS affects growth of *Salmonella* in macrophages late in infection, whereas NADPH oxidase has a role at the start of infection process (Cherayil and Antos, 2001). There were differences in recruitment of inflammatory cells, granuloma formation, or serum interferon γ levels between wild type and iNOS null mouse after *Salmonella* infection but both iNOS and NADPH oxidase are necessary for the maximum protection. (Mastroeni *et al.*, 2000;Vazquez-Torres *et al.*, 2000a). Indeed, recently it has been shown that *Salmonella* DNA bases are damaged by RNS in vivo (Richardson *et al.*, 2009).

1.3.2 Phagosomal killing by non-oxidative pathways

Cationic antimicrobial peptides

Cationic antimicrobial peptides are generally short and positively charged peptides which are an important first line of the host defense against various infections. Antimicrobial peptides are found in a variety of hosts including bacteria, insects, invertebrates, amphibians, birds, fishes and mammal including humans. Some antimicrobial peptides have potent and direct bactericidal activity and bacteria killing by antimicrobial peptides is often accompanied by the perimeabliztion of the bacterial membrane. In addition, the functions of CAMPs as immuno modulators have been recently revealed. Here we discuss two classes of the CAMP; CRAMP and defensins. CRAMP is found in mouse macrophage and neutrophil and defensins are the major CAMP in neutrophils.

Cathelicidine antimicrobial peptide (CRAMP)

CRAMP is ortholog of human LL-37. Given that the promoters of these orthologs are highly homologous and the proteins are more than 60% identical at the amino acid level, CRAMP is good model for the study of LL-37 regulation and function. Mouse CRAMP is made as a 172 residue pre-protein. Protease-mediated maturation yields a 34 amino acid CRAMP peptide with antimicrobial activity. CRAMP expression is detected in adult testis, spleen, stomach, and intestine, but not in brain, liver, heart, or skeletal muscle (Gallo *et al.,* 1997). CRAMP has an important role in innate immunity as evidenced by the fact that Group A streptococci can infect CRAMP knockout ($Cnlp^{-/-}$) mice more efficiently than wild type mice (Nizet *et al.*, 2001). Rosenberger et al also showed that

CRAMP is detected in bone marrow derived macrophages and is strikingly induced during *Salmonella* infection (Rosenberger *et al.*, 2004).

Defensins

Defensins are antimicrobial peptides found in most multicellular organisms. They are characterized by a β-sheet-rich fold with six disulfide linked cysteine residue (Ganz, 2003). Defensins are classified into α- or β- depending on how the six cysteines form the disulfide bonds. In many animals, the highest concentration of defensins (>10 mg/ml) is found in the granules of leukocytes. Paneth cells in the intestinal crypts also contain defensins-rich secretory granules that are released into the crypt lumen. The defensin concentration in the crypts can be as high as >10 mg/ml (Ayabe *et al.*, 2000). The distribution of defensins varies with species. Mice lack leukocyte defensins but produce several Paneth cell defensins and epithelial cell β-defensins (Eisenhauer and Lehrer, 1992). Rabbit alveolar macrophages have comparable amounts of α-defensins to rabbit neutrophils but there are no defensins in rabbit peritoneal macrophages (Ganz *et al.*, 1989). Human monocytes, macrophages and lymphocytes can produce detectable amount of defensins, but not as much as rabbit macrophages (Agerberth *et al.*, 2000). However, the direct action of defensins against bacteria within macrophages has not been elucidated.

Function of Lysosomal Proteases during bacterial infection

The autophage-lysosomal pathway of intracellular protein degradation is a non-selective bulk process (Kopitz *et al.*, 1990). More than 90% of all long lived proteins and a large fraction of short-lived proteins are degraded in the lysosome (Ahlberg *et al.*, 1985).

Apparently, engulfed bacterial proteins are also degraded upon phaogosome-lysosomal fusion. Lysosomal proteases also contribute to the killing of infected bacteria in the phagolysosome. Lysosomal proteases are largely classified into lysosomal cysteine proteases, lysosomal aspartic acid proteases, and serine proteases based on the catalytic site. Lysosomal cysteine proteases include Cathepsins S, B, C, H, K, L, K and asparagines endopeptidase. Cathepsin E and D are lysosomal aspartic acid proteases. The functions of the lysosomal proteases are complex and diverse. Here we confine our discussion to studies which relate to the immune system during bacterial infection. There are no clear reports that indicate the direct function of lysosomal cysteine proteases on bacterial survival in the phagosome. However, lysosomal cysteine proteases do regulate MHC class II antigen presentation. MHC molecules form heterodimer assemblies in the edoplasmic reticulum (ER) with the assistant of the invariant chain (Ii) chaperone molecule. Ii-MHC class II complex can travel to early endosomes to acquire antigens. The maturation of the endosome leads to activated lysosomal enzymes including lysosomal proteases. Ii is also degraded and leaving the class II associated chain peptide (CLIP) in the MHC class II peptide binding groove, preventing premature peptide loading. Removal of CLIP and loading of antigenic peptides is mediated by H-2M (Wolf and Ploegh, 1995;Hiltbold and Roche, 2002).

Lysosomal cysteine proteases, particularly cathepsin L and S, have an important role on MHC classs II antigen presentation. Cathepsin L is the most potent lysosomal proteases. Its degradation rate is 10 fold faster than any other cellular cysteine protease (Bohley and Seglen, 1992). Cathepsin S has a critical role in both Ii degradation and peptide generation. Cathepsin L is necessary to degrade Ii in cortical thymic epithelial cells(TECs)

but not in bone marrow derived antigen presenting cells (Nakagawa *et al.*, 1998). It was demonstrated that the inhibition of cathepsin S impairs MHC class II antigen presentation *in vivo* and *in vitro* (Riese *et al.*, 1996;Riese *et al.*, 1998). Even though both cathepsin L and S are dominant lysosomal proteases contributing to MHC class II antigen presentation, their functions do not overlap. Cathepsin S activity was detected in B cells, dendritic cells and macrophages, whereas cathepsin L is expressed by cortical TECs and macrophages (Nakagawa *et al.*, 1998;Nakagawa *et al.*, 1999). In macrophages, cathepsin S has an important role in the late stage of Ii degradation, but cathepsin L does not, even with a high level of mature cathepsin L (Beers *et al.*, 2003). This suggests that lysosomal proteases are non redundant and proteolytic activity is tightly regulated.

Salmonella efficiently avoid antigen processing and presentation by macrophages and dendritic cells. It was reported that antigen presentation in *Salmonella* infected macrophage is decreased PhoP-dependent. Later studies showed that PhoP is responsible for *Salmonella* evasion of lysosomal fusion. So it seems that PhoP decreases antigen presentation by evasion rather than by some direct effect. *Salmonella* antigen presentation in dendritic cells is suppressed by the expression of SPI-2 which did not have any effect on other dendritic cell functions. This suggests that SPI-2 may somehow directly control antigen presentation of dendritic cells. One can conceive that certain lysosomal proteases could target specific bacterial virulence protein.

Indeed, there are some examples of lysosomal proteases. Cathepsin D is a aspartic lysosomal protease and the most abundant lysosomal enzyme in rat liver (~10% of total lysosomal protein) (Dean and Barrett, 1976). Cathepsin D-deficient mice are less resistant to *Listeria monocytogenes* infection than wild type mice. Cathepsin D is capable

of degrading Listeriolysin O in the fibroblast phagosome and *Listeria* survives and replicate 10 times better in Cathepsin-deficient macrophages than in wild type macrophages (del Cerro-Vadillo *et al.*, 2006).

Another example of direct proteolysis of bacterial proteins was shown with elastases. Elastase is a serine protease that has an important role in bacterial infection in neutrophils and macrophages. The human neutrophil elastase is capable of efficiently degrading the virulence factors of *Shigella dysenteriae*. As a result, abrogation of neutrophil elastase allows *Shigella* to escape from the phagosome to the cytosol (Weinrauch *et al.*, 2002).

It has recently been reported that macrophage elastase (also known as matrix metalloproteinase 12 or MMP12) has direct bactericidal ability in vitro. *Mmp12*$^{-/-}$ mice show poor bacterial clearance and increased mortality when challenged with either Gram-positive or negative bacteria (Houghton *et al.*, 2009).

Lysosomal proteases are also capable of controlling immune signaling upon infection. The lysosomal cysteine protease, Cathepsin E, is exclusively present in immune cells. *CatE*$^{-/-}$ mouse are susceptible to bacterial infection due to the impaired TNF- α and IL-6 production (Tsukuba *et al.*, 2006). These abnormal immune responses in *CatE*$^{-/-}$ are caused by diminishing numbers of chemokine and N-formyl peptide receptors which lead to a defect in chemotaxis and cell adhesion(Tsukuba *et al.*, 2009).

Compared to the most potent antigen presenting dendritic cells, the less efficient antigen presenting cells, macrophages, produce dramatically higher amounts of lysosomal proteases including Cathepsin L, S, D, B and asparagine endopeptidase. The lack of complete protein degradation likely contributes to the superior antigen presentation ability of dendritic cells. On the other hand, it is also another clear indication that those

proteases contribute to bacterial killing and degradation in the phagosome during infection (Delamarre *et al.*, 2005).

In conclusion, lysosomal proteases work in a non-redundant manner and can directly kill bacteria or control the immune response. However, proteases that directly target a specific *Salmonella* protein still remain undefined.

1.3.3 Combinatory effect of proteases, cationic antimicrobial peptides and reactive oxygen species

Reactive oxygen species not only directly damage or kill engulfed microorganisms, but also function in immune regulation. For example, neutrophil mitogen activated protein (MAP) kinase activity is induced by H_2O_2 or the oxidant diamiade (Fialkow *et al.*, 1994). Induction of MAP kinase leads to the activation of transcription factors such as c-jun, which is involved in proinflammatory cytokine production (Qi and Elion, 2005). Microarray experiments performed on macrophages isolated from iNOS-/-, phox-/-, or double knockout mice also revealed that reactive oxygen or nitrogen intermediates help to orchestrate transcription by regulating different sets of genes (Ehrt *et al.*, 2001). Therefore it is clear that ROS and RNS have extensive effects beyond direct bacterial killing.

In neutrophils, one study demonstrated that the ROS generated during the oxidative burst can also act as a direct signal for protease activation (Reeves *et al.*, 2002). The authors of this work showed that the NADPH oxidase is stimulated by bacterial uptake and leads to the production of anionic molecules such as O_2^- or OH^- coincident with the entry of acidic granule contents to the phagosome. The negative charges on ROS are compensated by influx of H^+ or K^+ and the pH of the phagosome becomes neutral. These conditions

induce activation of the cationic granule proteases. The optimal pH for the phagocytic proteases including Cathepsin G and elastase is around 7. Unlike previous research, these results suggested that the neutrophil proteases have a direct role in killing the bacteria, while the ROS act by simply activating the proteases.

In contrast to what happens in the neutrophil, Rybicka *et al*. showed that NADPH oxidase negatively regulates protease activity in macrophages (Rybicka *et al.*, 2010). The expression of lysosomal proteases in macrophages is higher than that of dendritic cells. This is reasonable because dendritic cells are more potent antigen presenting cells than are macrophages. Activated NADPH oxidase is capable of depressing the reductive capacity of the phagosome. The active site of cysteine proteases such as Cathepsin B/L should be in the thiol form to initiate the catalytic cycle. Therefore NADPH oxidase inactivates cysteine Cathepsins through the decrease ability to reduce disulfides in the phagosome.

Rosenberger *et al* showed that *Salmonella* in macrophages are challenged with not only ROS but also antimicrobial peptides and proteases (Rosenberger *et al.*, 2004). These investigators report that *Salmonella* cell division is impaired during infection in macrophages resulting in filamentation. When macrophage proteases or production of ROS was inhibited, filamentation of the *Salmonella* was not seen *in vivo*. CRAMP could induce filamentation of *Salmonella* in vitro. Furthermore, a cooperative action of CRAMP and proteases on *Salmonella* was observed. Taken together, one must conclude that the various antimicrobial substances produced in macrophages orchestrate to kill infecting bacteria directly.

1.3.4 Neutrophil contribution during *Salmonella* infection

Neutrophils, also called polymorphonuclear neutrophils (or PMNs), are the first cells to respond to a *Salmonella* infection in submucosal areas. Neutrophils are rapidly recruited and release Tumor necrosis factor α (TNF-α) and produce RNS in *Salmonella* infected tissue (Kirby *et al.*, 2002;Rydstrom and Wick, 2007)). It has been generally accepted that PMNs are capable of killing *Salmonella* in vitro. However, there are still some unresolved issues regarding previous reports. *Salmonella* LT2, which is avirulent due to *rpoS* mutation, was used in one experiment showing *Salmonella* killing by PMNs (Stinavage *et al.*, 1989), whereas the other group used virulent *Salmonella* 14028 and showed the similar result (Fierer, 2001). There was also a direct study comparing the survival of *Salmonella* wild type and an *rpoS* mutant. In order to make sense of both reports, it is reasonable to conclude that PMNs eradicate *Salmonella* so efficient so presence of sigma S may be not the factor anymore. SPI-2 deletion mutant does not restore the virulence in neutrophil depleted mouse. Therefore neutrophil has no role during *Salmonella* systemic infection. In a study consistent with the above conclusion, it was shown that *Salmonella* grows better in neutropenic mice compared to normal mice (Vassiloyanakopoulos *et al.*, 1998). *Salmonella* shows neither evasion of phagocytosis or survival in the phagosomes of PMNs (Fierer, 2001). Slamonella pathogenesis island 2 mutnat or auxotrophic mutant showed impaired grown in neutrophil but their virulence is not recovered in neutrophil depleted mouse (Cheminay *et al.*, 2004). Combined with the neutropenic mouse experiment and the work of Cheminay et al., it can be concluded that neutrophils may have some functions during *Salmonella* infection but cannot suppress *Salmonella* systemic infection.

1.4 *Salmonella* Resistance to Oxidative Killing of Macrophages

In order to combat ROS, *Salmonella* encodes various superoxide dismutase, catalases, and peroxidases. *Salmonella* has four superoxide dismutases: SodA(Mn-SOD) and SodB(Fe-SOD) are cytoplasmic, while SodCI and SodCII are copper-zinc cofactor periplasmic SODs. In order to block the peroxidase activity completely in *Salmonella*, three catalases (KatE, KatG and KatN) and two alkyl hydroperoxide reductases (AhpC and TsaA) had to be deleted (Hebrard *et al.*, 2009).

1.4.1 ROS target

In addition to exogenous sources of superoxide, including phagocytic ROS, aerobically growing bacterial cells produce endogenous ROS. In *E. coli*, superoxide and hydrogen peroxide are produced by autooxidation of respiratory dehydrogenase (Messner and Imlay, 2002). The target of phagocytic superoxide is not known, but the cytoplasmic target of endogenous superoxide has been well studied. The first known targets of superoxide are exposed [4Fe-4S] clusters. One of the four irons in the $[4Fe\text{-}4S]^{2+}$ is coordinated by a water molecule while the others are coordinated with sulfur. Superoxide tends to be attracted to the catalytic iron atom. An attracted O_2^- univalently oxidizes the cluster to make $[4Fe\text{-}4S]^{3+}$. This oxidized cluster becomes unstable and degradation follows (Flint *et al.*, 1993). Enzyme inactivation caused by iron loss often leads to belongs block in the metabolic pathway. For example, Benov and Fridovich described an aromatic amino acid auxotrophy in mutants lacking cytoplasmic superoxide dismutase (Benov and Fridovich, 1999). O_2^- interferes with the production of erythrose-4-phosphate, which is essential for the first step in the aromatic amino acid biosynthetic pathway, by

the inactivation of the 1, 2-hydroxyethyl thiamine pyrophosphate intermediate in transketolase.

Hydrogen peroxide is also capable of oxidizing $[4Fe\text{-}4S]^{2+}$ clusters and releasing iron to produce an inactive form of $[3Fe\text{-}4S]^{+}$ (Jang and Imlay, 2007). The only ROS that can directly damage biomolecules is $HO\cdot^{-}$, which can be produced from hydrogen peroxide through the Fenton reaction. Millimolar levels of hydrogen peroxide are enough to mutagenize or kill most bacteria through the Fenton reaction (Imlay and Linn, 1988;Imlay *et al.*, 1988). When all the catalase and peroxidate enzymes in *E. coli* are deleted (referred to as *Hpx*$^{-}$), the concentration of endogenously produced hydrogen peroxide is around 1μM. This endogenously produced H_2O_2 is sufficient to damage DNA (Park *et al.*, 2005).

It has been shown that superoxide cannot easily penetrate the bacterial cytoplasmic membrane at neutral pH. However, O_2^{-} penetration improves at lower pH (Korshunov and Imlay, 2002). It can be postulated that phagocytic superoxide is also damaging cytoplasmic biomolecules to kill the bacteria. However, Craig *et al.* showed that SodCI acts independently of the cytoplasmic superoxide dismutases SodA and SodB during infection. This is a clear indication that whatever SodCI protects does not overlap with what cytoplasmic Sods protect (Craig and Slauch, 2009). Even though the periplasmic target of O_2^{-} has not elucidated, clearly the target of SodCI is located somewhere in the extracytoplasmic space. The function of SodA and SodB during *Salmonella* infection is quite obvious. The phagocytic environment is considered aerobic and *Salmonella* is aerobically respiring during growth in the host (Craig and Slauch, unpublished). A *Salmonella sodA sodB* double mutant cannot compete with wild type even in rich

laboratory medium when grown aerobically (Craig and Slauch, 2009). Moreover, the *sodA sodB* double mutant is attenuated in phox-/- mice that cannot produce phagocytic superoxide (Craig and Slauch, 2009). Thus, the cytoplasmic Sods are required to combat endogenously produced superoxide that is independent of phagocytic ROS.

The function of periplasmic SodCI or SodCII has not been fully elucidated. One of the challenges to answer this question is that no in vitro phenotype of the periplasmic *sodC* deletion mutant has yet been reported. The phenotype of *Salmonella* periplasmic *sodC* mutants is only observed in mouse during infection. One of the reasons could be the working concentration of superoxide. The concentration of superoxide in periplasm is theoretically the same as the environment because superoxide can cross the outer environment freely through the outer membrane porins. The typical amount of superoxide generated in vitro using the xanthine/xanthine oxidase system is less than 1μM for a minute or two (McCord and Fridovich, 1969). In contrast, it is estimated that the concentration of superoxide in phagosome could be 100μM steady state (Craig and Slauch, 2009).

1.4.2 *Salmonella* periplasmic superoxide dismutases

1.4.2.1 Regulation of *Salmonella* periplasmic superoxide dismutases

Salmonella has two periplasmic superoxide dismutases, SodCI and SodCII, but only SodCI is able to contribute to the virulence(Krishnakumar *et al.*, 2004;Fierer, 2001). What makes SodCI special during infection in the mouse? SodCI is encoded on functional Gifsy-2 prophage, whereas SodCII is chromosomally encoded. Thus, *sodCI* is horizontally transferred like other genes that contribute to *Salmonella* pathogenicity.

Consistent with this idea, SodCI is found only in highly virulent *Salmonella* serotypes (Fang *et al.*, 1999).

Transcription of *sodCI* is controlled by the two-component regulatory system PhoPQ and is induced ~17 fold in bacteria recovered from cultured macrophages or mouse spleens (Fang *et al.*, 1999;Golubeva and Slauch, 2006). The *sodCII* gene is controlled by RpoS and is also induced ~3-4 fold during infection of macrophages and mice, albeit to a lesser extent than *sodCI* (Golubeva and Slauch, 2006). However, differential gene expression cannot explain the differential contribution to virulence: a strain containing SodCI expressed from the *sodCII* locus was not significantly attenuated, whereas SodCII expressed from the *sodCI* locus did not contribute to virulence (Krishnakumar *et al.*, 2004). Thus, both proteins are apparently made during infection, suggesting that some physical difference between the two proteins allows SodCI, but not SodCII, to effectively combat phagocytic superoxide.

1.4.2.2 The controversy over SodC contribution to *Salmonella* virulence

The contribution of the periplasmic superoxide dismutase to *Salmonella* virulence was first shown by Fang and colleagues (Fang *et al.*, 1999;De Groote *et al.*, 1997). It was shown that a *sodCI* mutant is more sensitive to chemically produced superoxide and attenuated in a mouse virulence model compared to the wild type. But Fang *et al.* reported that both SodCI and SodCII are able to contribute to virulence in the mouse model. Subsequently, Sansone *et a.l* and Ammendola *et al.* also reported that both SodCI and SodCII are able to contribute to virulence (Sansone *et al.*, 2002;Ammendola *et al.*, 2005). In contrast, both the Bossi (Uzzau *et al.*, 2002) and Slauch (Krishnakumar *et*

al., 2004) labs published data showing that only SodCI, but not SodCII, has a role in virulence. It is interesting to note that all of the experiments suggesting a role for SodCII were performed using the same plasmid insertion allele of sodCII and in some cases, the exact same strain constructed by Fang group. The *sodCII* strain constructed by the Fang group likely yields a truncated SodCII and this may have a toxic effect during infection. Both Bossi and Slauch used clean deletion of the entire *sodCII* gene. The controversy was recently settled when Ammendola and coworkers, in collaboration with Fang (Ammendola *et al.*, 2008), showed that their original strain was flawed. It is now the consensus that only SodCI contributes to virulence during infection in the animal. In contrast, SodCII is not required during infection, even in the absence of SodCI.

1.4.2.3 Differences between SodCI and SodCII

While only SodCI can contribute to virulence during infection, data suggest that both proteins are produced in the animal. This suggests that the differential role in virulence is due to some difference in the two proteins. SodCI and SodCII share 60% identity at the amino acid level. Interestingly, the N-terminal 26 amino residues of these proteins share only 28% identity. I constructed hybrid proteins in which these distinctively different parts of SodC were switch. The properties of the hybrid proteins were analyzed, but no significant differences were observed (Krishnakumar *et al.*, 2007).

SodCI and SodCII also act similarly in various conditions such as diverse pH or the presence of chelators, and both are resistant to hydrogen peroxide (Krishnakumar *et al.*, 2004). SodCI is reported to have a 2.7 fold higher specific activity than SodCII. These two enzymes also differ in affinity for Cu and Zn (Gabbianelli *et al.*, 2004). However it

seems unlikely that subtle differences in enzymatic activity can completely explain the all or nothing phenotype observed during infection. Even overproduction of SodCII from the *sodCI* promoter does not complement a *sodCI* null phenotype. Moreover, even if SodCII accounted for only a fraction of the activity, one would expect to see a phenotype conferred by loss of SodCII in a *sodCI* null background. This is exactly what is observed with mutations in the cytoplasmic SODs, which protect against endogenously generated superoxide (Craig and Slauch, 2009). Null mutations in *sodA* confer no virulence phenotype, whereas mutants lacking SodB are attenuated ~4 fold. However, loss of SodA in a *sodB* null background confers a further 300-fold attenuation.

We have sought other properties, beyond enzymatic activity per se, to explain the inability of SodCII to protect against phagocytic superoxide. From work in our lab and that of others we know that SodCI is dimeric and protease resistant (Krishnakumar *et al.*, 2007;Ammendola *et al.*, 2008;Pesce *et al.*, 2000). Our lab has also discovered that SodCI is "tethered" within the periplasm by a non-covalent ionic interaction. In vitro, this is manifested as limited release by osmotic shock (Krishnakumar *et al.*, 2004;Krishnakumar *et al.*, 2007). SodCII is monomeric, protease sensitive, and released normally from the periplasm (Krishnakumar *et al.*, 2007). Our lab has also found that SodC from *Brucella abortus*, when expressed from the *sodCI* locus, was able to complement, resulting in full virulence. This enzyme is monomeric, released by osmotic shock, but protease resistant, suggesting that this latter property is critical in the phagocyte (Krishnakumar *et al.*, 2004;Krishnakumar *et al.*, 2007). However, tethering within periplasm is unique property as a periplasmic protein especially SodCI binding to a target is ionic interaction therefore role of tethering is examined in this report.

1.4.3 Role of catalases and peroxidases in *Salmonella* virulence

In aerobically growing cell, superoxide is produced adventitiously by electron carrying redox enzymes. Cytoplasmic superoxide is dismuted by SodA and SodB to hydrogen peroxide. This hydrogen peroxide is eliminated by catalases (KatE and KatG) and alkyl hydroperoxide reductase (AhpC) to water in *E. coli*. If these endogenous reactive oxygen species are not properly scavenged, they cause oxidation of $[4Fe\text{-}4S]^{2+}$ clusters, which release iron. The free Fe reacts with H_2O_2 via the Fenton reaction to create hydroxyl radical, which reacts in a diffusion limited manner with any biological molecule including DNA (Imlay, 2008;Imlay, 2003). Therefore it is conceivable that exogenous hydrogen peroxide produced from superoxide by SodC may be able to damage cytoplasmic biomolecules. Superoxide is unable to penetrate the bacterial membrane at neutral pH because it is anionic, but hydrogen peroxide can penetrate the membrane. The *Salmonella*-containing macrophage phagosome is acidic, so superoxide is potentially protonated and able to penetrate the membrane where it could be dismuted (puche Aranda *et al.*, 1992). However, it is difficult to test the effects of periplasmic SodC-mediated hydrogen peroxide in *Salmonella* virulence. *Salmonella* has three catalases (KatE, KatG and KatN) and two alkyl hydroperoxide reductases (AhpC and TsaA). The virulence of *katE* and *katG* single or double mutants are not attenuated in macrophages or the mouse model. Only an *hpxF* (*katE katG katN ahpC tsaA*) mutant is attenuated in macrophages or mice during infection (Hebrard *et al.*, 2009). Unlike *E.coli*, growth phenotypes caused by endogenous hydrogen peroxide in the *Salmonella ⊿*Kat (KatE, KatG and KatN), *⊿*Ahp (AhpC and TsaA) and *⊿hpxF* are very similar. In contrast, the *hpxF*$^-$ strain is more sensitive to exogenous H_2O_2 than the Kat$^-$ or Ahp$^-$. Therefore the

enzymes involved in detoxifying H_2O_2 may be important to surviving in macrophage and mouse, but it is difficult distinguish whether these defects are caused by exogenous or endogenous hydrogen peroxide. This is analogous to the fact that a *sodAB* double mutant is attenuated both in aerobic rich medium and the mouse model (Craig and Slauch, 2009).

SoxRS

It was found that redox cycling agents such as paraquat induce MnSOD synthesis in *E. coli* (Hassan and Fridovich, 1977). It was subsequently discovered that this regulation is mediated by the SoxRS system. The SoxR sensor acts as a homo-dimer with a single $[2Fe\text{-}2S]^{+}$ cluster at the interface. Superoxide or redox agents oxidize the cluster to the +2 state, causing SoxR to activate transcription of *soxS*, encoding a transcriptional activator that turns on not only MnSOD but also some putative cluster repair enzymes, drug efflux pumps and other factors that confer resistance to these compounds (Imlay, 2008). However, SoxRS regulation by these redox cycling agents remains controversial. For example, redox recycling agents are able to regulate *Pseudomonas soxS* under anaerobic conditions. Therefore the simple model that superoxide directly induces *soxRS* is likely not true (Dietrich *et al.*, 2006). Moreover, SoxS is required for *Salmonella* survival in paraquat but not during infection in mice (Fang *et al.*, 1997). This is further evidence that phagocytic superoxide is likely not gaining access to the bacterial cytoplasm.

OxyR

The *oxyR* locus was first identified in a *Salmonella* mutant that is hypersensitive to hydrogen peroxide (Christman *et al.*, 1989). OxyR regulates more than 20 genes in *E.*

coli including catalases, *fur*, *dps* and the FeS cluster assembly system. *Salmonella* oxyR mutant does not attenuated during infection in mouse (Taylor *et al.*, 1998). However, null mutations in the *oxyR* regulated genes *dps* or *mntH* do attenuate *Salmonella* virulence (Fields *et al.*, 1986;Zaharik *et al.*, 2004;Halsey *et al.*, 2004). It seems like attenuated genes were not only regulated by *oxyR*. For example Fur is required for the full activation of Dps (Yoo *et al.*, 2007).

1.5 *Salmonella* Resistance to Non-Oxidative Killing of Macrophages

PhoPQ

PhoPQ is a two-component regulatory system that plays an important role in *Salmonella* virulence. It consists of senor kinase PhoQ and cytoplasmic regulator PhoP. Phosporylated PhoP binds to and regulates more than 200 genes known as either *phoP* activated (pag) or *phoP* repressed (prg) genes (Fields *et al.*, 1986;Monsieurs *et al.*, 2005). The virulence of *phoP* or *phoQ* gene deletion mutants is highly attenuated in the mouse model. A constitutive *phoQ* mutation also attenuates as much as a *phoP* deletion in the mouse model (Fields *et al.*, 1986;Miller and Mekalanos, 1990). This suggests that precise regulation of the regulon during infection is necessary. A *phoP* mutant is highly sensitive to cationic antimicrobial peptides (CAMPs) including mammalian defensins, frog derived magainin 2, insect derived melittin and polymyxin B because PhoPQ regulates genes responsible for CAMP protection from *Salmonella* such as *pmrAB* or *mig-14* (these will be explained later chapter).

The transcription of *pags* is activated in micromolar $Mg2^{+}$ and repressed in millimolar $Mg2^{+}$. Besides $Mg2^{+}$, PhoP responds to $Ca2^{+}$ and $Mn2^{+}$ but not $Ni2^{+}$,$Cu2^{+}$, $Co2^{+}$ or $Ba2^{+}$ (Garcia *et al.*, 1996). The other PhoP activating signals are acidic pH. These are the conditions present in the macrophage phagosome that presumably lead to activation of the regulon. In addition to *pags* and *prgs*, PhoPQ also controls expression of a two-component system that controls SPI2, SsrA(SpiR)/SsrB (Bijlsma and Groisman, 2005).

PmrAB-dependent protection

PhoPQ activates the second two component regulatory system, PmrAB, which is largely responsible for resistance to cationic antimicrobial peptides. PmrAB can be activated by high Fe, acidic pH, and cationic antimicrobial peptides, or PhoPQ via PmrD. PmrA-regulated genes are activated both in the lumen of the intestine and in the spleen during infection in mice. The PmrAB regulon is mainly activated via PhoPQ in the mouse model even though PmrAB can be regulated independently of PhoP *in vitro* (Merighi *et al.*, 2005).

The primary role of the PmrAB regulon is to alter the structure of LPS, including the removal of negative charges, to resist antimicrobial peptides. (Gunn, 2008). Products of the *pmrHFIJK* and *pmrE* genes are responsible for addition of 4-aminoarabinose (Ara4N) to the lipid A (Gunn *et al.*, 2000) so the LPS more positively charged. PmrC is responsible for phosphoethanolamine (pEtN) mediated lipid A (1-phosphate) modification whereas CptA modifies the LPS core (Heptose I phosphate) with the addition of pEtN (Lee *et al.*, 2004;Tamayo *et al.*, 2005). PmrG dephosphorylates heptose II in the LPS core (Nishino *et al.*, 2006).

Salmonella pmrAB mutant strain is attenuated in a mouse competition assay only when infected orally. The *pmrA* mutant competes evenly with wild type when infected by the intraperitoneal route (Gunn *et al.*, 2000). The reason for this discrepancy has not been resolved. It is interesting to consider that both *pmrA* and *phoP* are highly induced in both macrophages and the small intestine (Merighi *et al.*, 2005). Therefore, differential environmental regulation of these two systems cannot explain their differential role in virulence. One hypothesis is that different types of antimicrobial peptides are expressed in different tissues and these two systems differentially protect against the different peptides.

The Periplasmic Stress Response

One neglected aspect of *Salmonella* survival during infection is the role of the periplasmic stress response regulators. *Salmonella* overcomes numerous stresses in the phagosome including ROS, antimicrobial peptide, lysozysome and so on, all of which are extracellular and not capable of penetrating the bacterial cell in most cases. Therefore, it is easy to postulate a connection between phagosomal stress and periplasmic stress in *Salmonella*. There are the two major periplasmic stress response regulators, sigma E and CpxRA. Most of studies regarding sigma E and CpxRA are done in *E. coli*, so the descriptions below pertain to *E. coli* unless otherwise specified.

Sigma E

Sigma E is encoded by the *rpoE* gene, which was discovered as a regulator of the periplasmic protease DegP (HtrA) (Lipinska *et al.*, 1988). RpoE is essential in *E. coli* but

it is not essential in *Salmonella* (De Las *et al.*, 1997). The RpoE regulon is activated by ethanol and heat in vitro (Erickson and Gross, 1989). The *rpoE* gene is in an operon with *rseABC*. RseA is an inner membrane protein and the N terminal domain of RseA interacts with sigma E in the cytoplasm while the C terminal domain interacts with RseB in periplasm. DegS is a periplasmic protease with a so-called PDZ domain (Erickson and Gross, 1989;Missiakas *et al.*, 1997). When outer membrane assembly is keeping up with cell demand and there are few misfolded proteins in periplasm, DegS is inactive and RseAB and RpoE form a complex. In this state, RpoE is bound to RseA and thereby sequestered. Under periplasmic stress, assembly of outer membrane proteins can slow, exposing the C-terminus of beta barrel protein such as OmpC (consensus sequence: YXQ) to the periplasm where they are detected by the PDZ domain of DegS. This activates DegS, leading to cleavage of the periplasmic domain of RseA releasing bound RseB (Ades *et al.*, 1999;Walsh *et al.*, 2003). Next, the inner membrane protease RseP cleaves the cytoplasmic domain of RseA and as a result sigma E and the cleaved cytoplasmic part of RseA is released in the cytoplasm. The released sigma E and RseA complex binds to SspB, which delivers the RseA protein to the cytoplasmic AAA+ protease ClpXP leading to sigma E release (Flynn *et al.*, 2004). The released sigma E is able to function as sigma factor activating at least 43 genes including periplasmic proteases and folding proteins in *E. coli* (Dartigalongue *et al.*, 2001).

Function of sigma E in *Salmonella* virulence

A *Salmonella rpoE* mutant is sensitive to antimicrobial peptides and reactive oxygen species generating agents (Humphreys *et al.*, 1999;Testerman *et al.*, 2002). The *rpoE*

mutant is attenuated in the mouse by both intraperitoneal and oral route. Whether this attenuation is due to sensitivity to antimicrobial peptides was never directly tested.

As mentioned above, the first gene known to be regulated by sigma E encodes the periplasmic protease HtrA. HtrA is a periplasmic serine protease and *htrA* mutants show defective growth in hydrogen peroxide but not in polymyxin (Humphreys *et al.*, 1999;Mutunga *et al.*, 2004;Mutunga *et al.*, 2004). An *htrA* deletion mutant cannot survive and replicate in wild type mice or and macrophages but is virulent in $gp97^{-/-}$ (deletion of one of the subunits of NADPH oxidase) mice (Strahan *et al.*, 1992;Mutunga *et al.*, 2004). SurA and FkpA are also sigma E regulated proteins that have a role in protein folding in periplasm. A *surA* mutant is attenuated in mice but an *fkpA* mutation alone is not sufficient to attenuate infection (Sydenham *et al.*, 2000;Humphreys *et al.*, 2003).

CpxRA

CpxRA is a two component regulatory system that responds to extracytoplasmic stress. CpxRA regulate more than 100 genes including those encoding the periplasmic protease HtrA, the protein disulfide bond oxidoreductase DsbA, the periplasmic proline isomerases PpiA and PpiD, and also the *cpxRAP* operon (Sydenham *et al.*, 2000;De *et al.*, 2002;Raivio and Silhavy, 1999). Note that some of these genes are also regulated by sigma E and the sigma E and Cpx are capable of compensating for one another when either one is inactivated (Sydenham *et al.*, 2000;Humphreys *et al.*, 2004). CpxP binds to the periplasmic domain of CpxA and presumably down regulates phosphorylation of CpxR. The periplasmic stress response was first observed in *E. coli* by overexpressing pilin proteins. When misfolded pilin proteins are induced, CpxP binds to the pilin and

dissociates from CpxA. The CpxP-pilin complex is subsequently degraded by HtrA. The dissociated CpxA phosphorylates CpxR and phosphorylated CpxR regulates the members of the CpxR regulon (DiGiuseppe and Silhavy, 2003;Raivio *et al.*, 2000;Raivio *et al.*, 1999;Isaac *et al.*, 2005). Both a *cpxA* deletion mutation and *cpxA**, which constitutively induces the Cpx regulon, attenuates *Salmonella* during mouse infection. However, it was reported that a cpxR mutation does not attenuate (Humphreys *et al.*, 2004). This discrepancy has not been resolved.

Alternative defensive systems that protect against antimicrobial peptides

In addition to the PhoPQ/PmrAB dependent modifications of LPS, *Salmonella* have alternative ways to defend against antimicrobial peptides. The first reported alternative system is the outer membrane protease PgtE. The *pgtE* gene is regulated by PhoP and *Salmonella* that over express PgtE are more resistant to the cationic antimicrobial peptide C18 because they cleave it (Guina *et al.*, 2000). Ramu *et al.* showed that *Salmonella* PgtE is also capable of activating macrophage proMMP-9 (gelatinase B). Interestingly *pgtE* mutants are attenuated during mouse infection only in the first day of infection (Reeves *et al.*, 2002).

The second system is *mig-14*, which encodes a horizontally transferred membrane associated protein that contributes to mouse infection in vivo (Valdivia *et al.*, 2000). The *mig-14* gene also regulated by PhoPQ and *mig-14* mutants show increased sensitivity to cationic antimicrobial peptides such as polymyxin B or CRAMP. The most recent report suggested that CRAMP binds to Mig-14 and this may inhibit CRAMP penetration through the bacterial membrane (Brodsky *et al.*, 2002;Brodsky *et al.*, 2005).

Barker and coworkers found that the O_2 consumption in stationary *Salmonella* or *E. coli* plummeted when they encountered antimicrobial peptides (Barker *et al.,* 2000). When the bacteria were supplemented with formate immediately before treatment with the antimicrobial peptides, they survived better. Formate dehydrogenase deletion strains lose the formate dependent protection (Barker *et al.*, 2000). This suggests that antimicrobial peptides somehow affect the respiratory chain, but the mechanism of this protection is not known.

Lastly, an interesting protein that potentially could function in *Salmonella* virulence is ecotin. Ecotin is a periplasmic protease inhibitor originally identified in *E. coli* (Lipinska *et al.*, 1988;Maurizi, 1992). Ecotin exists in the wide variety of bacterial species including *Salmonella*. The ecotins from difference species are highly conserved. Purified ecotin from various organisms are all capable of inhibiting activity of various proteases including neutrophil elastase, Cathepsin G, and trypsin. The role of ecotin during infection in vivo has not been investigated but ecotin is clearly able to protect bacterial cells from neutrophil elastses in vitro (Eggers *et al.*, 2004).

Chapter 2: *Salmonella* Periplasmic Superoxide Dismutase, SodCI, is Non-Covalently Tethered to Peptidoglycan

2.1 Introduction

Salmonella survival and replication in macrophages is required for systemic infection. In the macrophage, *Salmonella* encounters various antimicrobial substances including superoxide and its derivative ROS. *Salmonella* produces two periplasmic superoxide dismutases, SodCI and SodCII. Only SodCI, but not SodCII, is able to contribute to virulence during infection in mice despite the two proteins have the same catalytic function (Krishnakumar *et al.*, 2004). Transcription of the two genes is different. The *sodCI* gene is regulated by the PhoPQ two component regulatory system, whereas *sodCII* is regulated by sigma S (Golubeva and Slauch, 2006). However, by swapping the open reading frames, our lab showed that the differential contribution during infection is not because of differences in regulation of the two genes but because of the inherent differences in the two proteins (Krishnakumar *et al.*, 2004).

Among the reported differences between the two proteins, sensitivity to proteases and "tethering" have been implicated in virulence. SodCII is released by osmotic shock like any other periplasmic protein but SodCI is somehow tethered within the periplasmic space (Krishnakumar *et al.*, 2004). Similarly, when *Salmonella* is treated with cationic antimicrobial peptides, which are known to release periplasmic proteins, SodCII is preferentially released, while SodCI is not (Kim *et al.*, 2010).

SodCI tethering within the periplasm of *Salmonella* apparently contributes to protecting the enzyme in the phagosome. SodCII cannot contribute to virulence in wild type mice.

However, when SodCII was protected from release, mediated by antimicrobial peptides, it could facilitate survival in macrophages (Kim *et al.*, 2010). This suggests that tethering of SodCI within the periplasm is important for *Salmonella* virulence.

The nature of tethering has not been elucidated, but there were indirect indications that help to pinpoint the tethering target. First, SodCI remains tethered even when over expressed. This suggests that SodCI binds to something abundant. Second, SodCI is tethered in both *E. coli* and *Salmonella*, so SodCI binds something that is common between these two species. Third, SodCI can be released by a high salt wash after osmotic shock (Krishnakumar *et al.*, 2004;Krishnakumar *et al.*, 2007). This indicates that SodCI tethering within the periplasm is mediated bynon-covalent bonding, likely an ionic interaction. Based on the facts above, we hypothesized that SodCI might bind to the peptidoglycan (PGN). In this chapter, we analyzed the interaction between SodCI and *Salmonella* PGN.

2.2 Materials and Methods

Media and reagents

Bacteria were grown in Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter), with 15 g of agar per liter added for solid medium. The concentrations of the antibiotics used were as follows: ampicillin and and tetracycline, 25 µg/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Fisher, *Bacillus Subtilis* PGN, *Staphylococcus* PGN and chitin was purchased from Sigma.

Strain construction

His tagged monomeric SodCI (mSodCI) was constructed starting with the previously built monomeric SodCI sequence (Krishnakumar *et al.*, 2004) This gene was amplified using primers (5' primer, CGA GGT AAC ATA TGA AAT ACA C; and 3' primer, TTG CTC GAG TTT CTC AAT GAC AC) that introduced an NdeI site at the start codon and an AvaI site at the 3' end, replacing the stop codon. This fragment was cloned into the pET21b plasmid, resulting in the *sodCI* ORF being in frame with 6xHis-encoding sequences at the 3' end. This plasmid was introduced into an otherwise *sod* null strain that produced the T7 polymerase under the control of LacI. Construction of SodCI-6His and phoA-6His were described previously(Kim *et al.*, 2010).

Protein purification

SodCI-6XHis, mSodCI-6His and PhoA-6His were purified using Ni columns. Cells containing the appropriate construct were grown overnight. The cells were spun down, resuspended in 20 mM sodium phosphate (pH7.4), and disrupted by passage through a French pressure cell. The cell lysate was loaded onto a Ni column (GE health care). The column was washed with 20 mM sodium phosphate buffer containing 15 mM imidazole. The Ni bound proteins were eluted with increasing concentrations of imidazole solution in 20 mM sodium phosphate buffer. Fractions were checked by running SDS-PAGE and Western blot analysis. Purified His-tagged proteins were dialyzed in 10 mM sodium phosphate with 0.15 M NaCl. Purification of native SodCI and SodCII was as described previously (Krishnakumar *et al.*, 2007).

Purification of *Salmonella* PGN

Salmonella PGN was purified based on the procedure of Shi-Yan *et al* (Li *et al.*, 2004). *Salmonella* 14028 was grown over night in 1liter LB then washed and resuspended in 15 ml of ice cold water. The same volume of 8% sodium dodecyl sulfate (SDS) was added to the cell suspension drop wise with constant stirring and then the mixture was boiled for 30 minutes. After the incubation in room temperature overnight, the lysates were pelleted by ultracentrifugation (100,000 g for 20 mins) and the pellet was resuspended in 2 ml of deionized water. The ultracentrifugation and resuspending process was repeated 10 times. The purified *Salmonella* PGN was resuspended in 1 ml deionized water. The resdual SDS was measured using a methylene blue assay until the concentration was below 1 μg/ml (Hayashi, 1975). The purified PGN was sonicated briefly before an each protein and peptidoglycan binding assay.

Protein-PGN binding assay and Western blot

To measure the binding of purified SodC proteins to PGN, approximately 1 μg of a given protein was mixed with 30 μl of purified *Salmonella* PGN. After one hour incubation at 4 ℃, the sample was centrifuged at 100,000 g for 20 mins to pellet the PGN. The supernatant was collected. The pellet was washed by adding 500 μl of 10 mM sodium phosphate buffer to tube and mixing gently. The sample was centrifuged again for 20 mins at 100,000 g. The supernatant was collected and concentrated to 50 μl in a SpeedVac concentrator (Savant). This fraction was considered the wash fraction. The remaining pellet was resuspended in 50 μl of the same buffer and considered the pellet

fraction. Control experiments without added PGN were performed identically to that described above except that an equivalent amount of buffer was added instead of PGN. To test SodCI binding to chitin, approximately 1 ug of SodCI was mixed with 0.3% (w/v) of chitin in 10 mM sodium phosphate buffer. After incubation at 4C for an hour, the sample was processed as described above. For binding assays with PGN from species other than *Salmonella*, 1 mg of PGN was used with the remaining steps as above. Each collected fraction was mixed with 5 x SDS loading buffer and boiled for 10 mins prior to SDS electrophoresis and Western blot analysis performed as describe previously (Kim *et al.*, 2010).

Isothermal titration calorimetry

All experiments were conducted using a MicroCal VP-ITC titration calorimeter preincubated to 25^{o}C for at least one-hour prior to the start of experiments. SodCI served as the ligand. SodC protein was at a final concentration of 10 µM in 20 mM sodium phosphate. The final pH was around 7.4. *Salmonella* PGN (OD 208 ~0.3) was used as a binding partner. The 1.4 ml of *Salmonella* PGN was loaded with a blunt-end needle attached to a 5 ml Hamilton pipette making sure to introduce no air bubbles into the sample cell. Likewise, the injection syringe was filled and expelled with the SodCI solution twice prior to finally being filled and made free of any air bubbles. Experimental parameters used with the VP-ITC were 28 x 10 µl injections with 5 minute spacings, 300 rpm stirring speed, and a reference power of 1 µcal/s.

2.3 Results

SodCI binds to *Salmonella* PGN

In order to test binding of SodCI to peptidoglycan, we purified His tagged SodCI, His-tagged monomeric SdoCI (mSodCI) (Krishnakumar *et al.*, 2004)and, as an additional control, His-tagged PhoA. The mSodCI does not tether to periplasm (Krishnakumar *et al.*, 2004). *Salmonella* PGN was also purified. To perform the binding assay, purified protein and PGN was incubated together for an hour at 4℃ then the PGN was pelleted by ultracentrifugation. The supernatant was saved. The pellet was gently washed and the PGN again pelleted by ultracentrifugation. The resulting supernatant was considered the wash fraction. The pellet was suspended in the equivalent amount of 10 mM sodium phosphate buffer. Control experiments were performed without added PGN. Each sample was mixed with SDS loading buffer and boiled. Proteins were separated by SDS-PAGE and an immunoblot was performed. Since PGN is found in the pellet after ultracentrifugation, we hypothesized that if a protein binds to PGN, the protein will be found in the pellet with the PGN. If a protein does not bind to PGN it will remain in the supernatant.

Figure 2.1 shows that SodCI, in the absence of PGN was primarily in the supernatant, although we routinely recovered some protein in the pellet. However, in the presence of PGN most of the SodCI was found in the pellet. This suggests that SodCI binds to *Salmonella* PGN.

To confirm this binding is exclusive to the SodCI, we tested PGN binding of His-tagged PhoA and His-tagged mSodCI. PhoA served as a general periplasmic protein control.

Monomeric SodCI (mSodCI) is the derivative of SodCI with two amino acid changes in the dimeric interface. This mSodCI is released by osmotic shock like any other periplasmic protein (Krishnakumar *et al.*, 2004). Both PhoA and mSodCI were found primarily in the supernatant or wash fraction even in the presence of *Salmonella* PGN (Fig.2.1). These data suggest thatbinding to *Salmonella* PGN is exclusive to SodCI.

Narrowing down SodCI binding site on *Salmonella* PGN

Next, we tried to determine what part of PGN was recognized by SodCI. To test if the N-acetylglucosamine in PGN is the target of SodCI binding, we used chitin, which is an N-acetylglucosamine polymer. SodCI did not pellet with chitin so SodCI does not seem to bind to N-acetylglucosamine. While the PGN of *Salmonella* and the Gram-positive *Staphylococcus* have the same carbohydrate structure, there are differences in the structure of the peptide and crosslink. When *Staphylococcus* PGN was used in our assay, SodCI was found mostly in the supernatant and wash fractions, but not in the pellet. This suggests that SodCI recognition of *Salmonella* PGN involves the peptide portion of the molecule.

To exclude the possibility that SodCI binds to something other than PGN per se that contaminates the *Salmonella* PGN, we measure binding to *Bacillus* PGN. Both the carbohydrate protion and peptide in Bacillus PGN are the same as in *Salmonella* PGN. This includes the *meso*-diaminopimelic acid at the third position of the peptide. When SodCI was tested with Bacillus PGN, SodCI was found mostly in the pellet. Thus, SodCI binds something in common between *Salmonella* and *Bacillus* PGN.

SodCI binds *Salmonella* PGN as confirmed by ITC

Finally we tested SodCI binding to *Salmonella* PGN using Isothermal Titration Calorimetry (ITC). The results (Fig.2.3) indicate that SodCI interacts with purified PGN in a concentration dependent manner. In contrast, no heat was released by the addition of PGN to buffer alone. Further controls are required to confirm these results.

2.4 Discussions

The periplasmic protein SodCI is not released by osmotic shock (Krishnakumar *et al.*, 2004). This is a unique characteristic for a periplasmic protein. Indeed, the conventional definition of a periplasmic protein is that it is released by osmotic shock. SodCI tethers within the periplasm even when it is over expressed from a plasmid. It is tethered in both *Salmonella* and *E. coli*. More importantly, SodCI can be released by high salt after osmotic shock, or by lysozyme treatment to create protoplasts. These results suggest that SodCI binds something abundant, likely peptidoglycan, via an ionic interaction. Here we show that SodCI specifically binds to *Salmonella* peptidoglycan. There are only a few reported peptidoglycan binding proteins. One is the murein degrading enzyme lytic transglycosylase 70 (Slt70), which catalyzes the cleavage of β-1,4 glycosidic bonds between N-acetylglucosamine and N-acetylmuramic acid (Holtje *et al.*, 1975;Walderich and Holtje, 1991). This protein is also not released by osmotic shock (Holtje *et al.*, 1975). The other reported peptidoglycan biosynthetic enzymes, which might be expected to bind peptidoglycan, contain transmembrane domains and hence are buried in the membrane. These proteins include peptidoglycan-associated lipoprotein (Pal) (Bouveret *et al.*, 1999) and flagella motor protein (MotB) (Hizukuri *et al.*, 2009) from *E. coli* and RmpM from

Neisseria meningitides (Grizot and Buchanan, 2004). However, *Salmonella* SodCI does not fall into any of the above categories. The *Drosophila* peptidoglycan recognition protein (PGRP-LE) is an innate immune system receptor. The interaction of this protein with bacterial to peptidoglycan has been carefully studied by Lim *et al.* (Lim *et al.*, 2006). PGRP-LE recognizes *meso*-diamiopimelic acid (DAP) containing peptidoglycan. Binding takes place at the dimeric interface of the PGRP-LE homodimer. Interestingly, SodCI is also dimeric. Moreover, mutations of SodCI that disrupts the dimer interface, resulting in an enzymatically active monomer, also lead to loss of tethering (Fig. 2.1). A simple explanation is that, like PGRP-LE, SodCI binding to peptidoglycan takes place at the dimer interface. However, our lab has now narrowed the region of SodCI that is required for tethering and it is somewhat removed from the dimer interface. Either the monomeric SodCI is conformationally altered in the binding region, or both binding domains of the dimer need to be engaged in order to have sufficient interaction with the cell wall. The prominent difference between *Salmonella* and *Staphylococcus* PGN is the third amino acid in the peptide, *meso*-DAP versus D-lysine. SodCI binds to *Bacillus* PGN which also contains *meso*-DAP. Considering the fact that SodCI tethering is apparently an ionic interaction (Krishnakumar *et al.*, 2007), the extra carboxylate group on *meso*-DAP would be the most probable binding site for SodCI. Therefore, SodCI binding to the *meso*-DAP residue in PGN needs to be studied further.

2.5 Tables and Figures

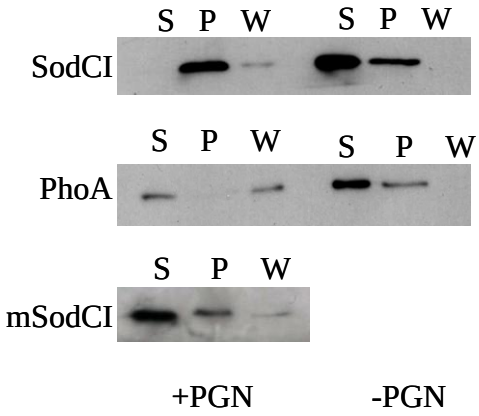


Figure 2.1 SodCI specifically binds to *Salmonella* PGN. PGN binding assay was shown with Western blot analysis. Each purified SodCI, phoA and monomeric SodCI is incubated with or without *Salmonella* PGN. Supernatant (S) after 1st centrifugation (100,000Xg) was saved. The remaining pellet was washed gently washed with 10mM sodium phosphate pH 7.4 then another supernatant (W) and pellet (P) were obtained after 2nd centrifugation (100,000Xg).

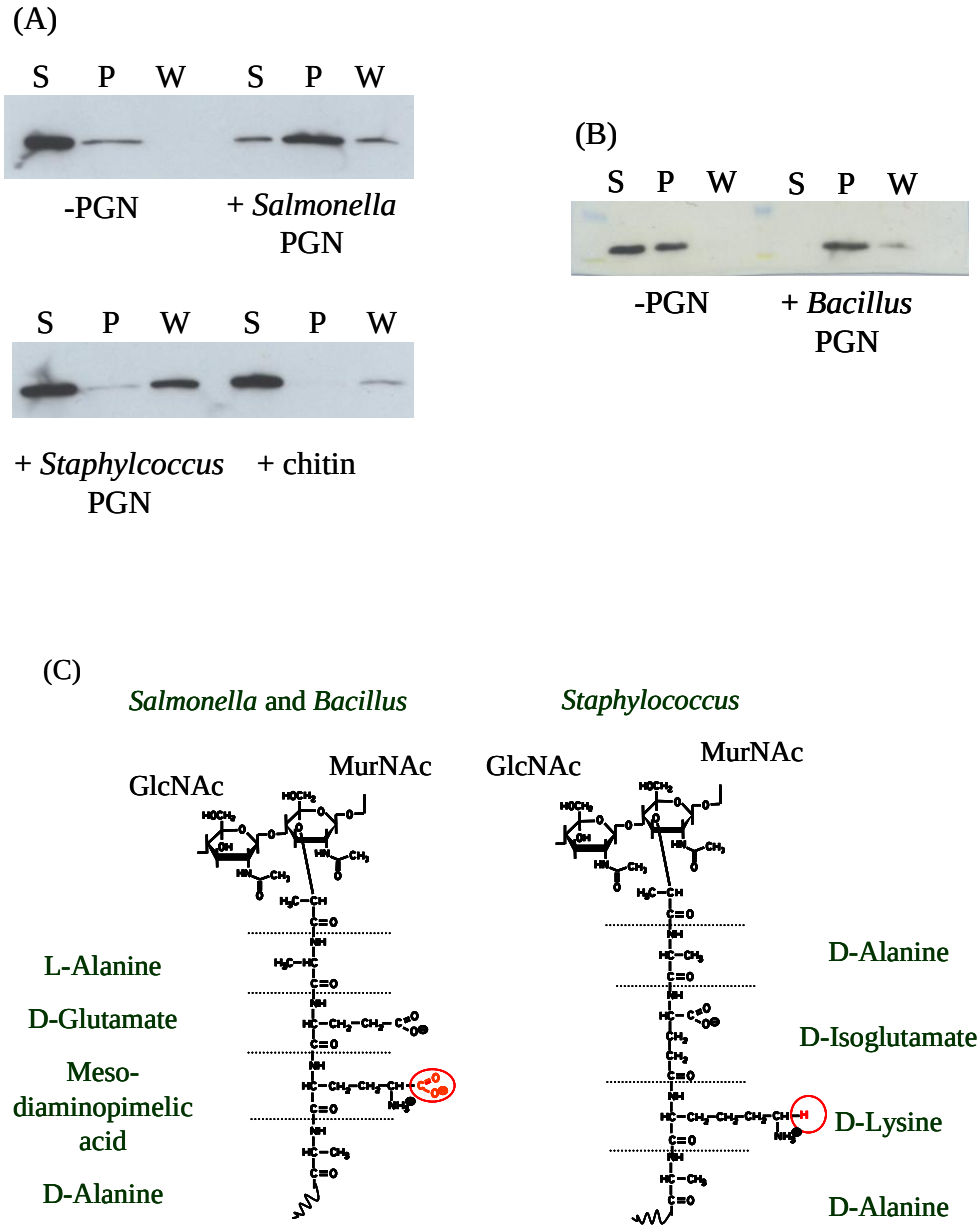
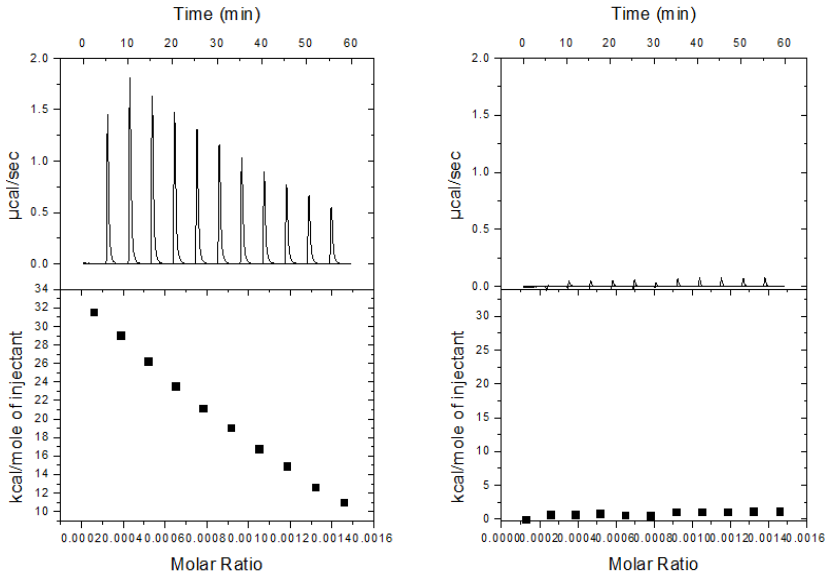


Figure 2.2 SodCI binds to peptide region of the *Salmonella* PGN. SodCI and *Staphylococcus* PGN or chitin binding was tested. SodCI binding experiment with or without *Salmonella* PGN were served as control (A). *Salmonella* binds to *Bacillus* PGN. Purified SodCI and Bacillus PGN was used to test binding (B). Representative structure of a unit of *Salmonella* , Bacillus subtilis and Staphylococcus PGN (C).



SodCI + *Salmonella* PGN Buffer + *Salmonella* PGN

Figure 2.3 SodCI binding to *Salmonella* PGN was confirmed by isothermal titration calorimetery. Purified SodCI and *Salmonella* PGN was tested (left panel). Buffer and *Salmonella* PGN was tested as a control (right panel).

Chapter 3: Protecting against Antimicrobial Effectors in the Phagosome Allows SodCII to Contribute to Virulence in *Salmonella enterica* Serovar Typhimurium

3.1 Introduction

Survival in macrophages is essential for *Salmonella enterica* serovar Typhimurium (Richter-Dahlfors *et al.*, 1997;Mastroeni *et al.*, 2009). In the phagosome, *Salmonella* is challenged with a plethora of antimicrobial substances, including superoxide, produced by NADPH oxidase, cationic antimicrobial peptides (CAMPs), which disrupt bacterial membranes, and phagocytic proteases (De Groote *et al.*, 1997;Radtke and O'Riordan, 2006). These oxidative and non-oxidative effectors act to inhibit or kill invading bacteria *(Rosenberger et al., 2004),* but direct mechanistic interactions among these effectors in vivo are largely speculative.

Salmonella resistance to the oxidative burst of phagocytes requires periplasmic Cu/Zn-cofactored superoxide dismutase (SodC) (Craig and Slauch, 2009). *Salmonella* serovar Typhimurium strain 14028 produces two periplasmic superoxide dismutases, SodCI and SodCII. We and others have shown that only SodCI contributes to virulence during infection in the animal. In contrast, SodCII is not required during infection, even in the absence of SodCI (Krishnakumar *et al.*, 2004). It is clear that SodCI specifically protects against phagocytic superoxide; there is no other role for the enzyme (Craig and Slauch, 2009;Krishnakumar *et al.*, 2007).

Both proteins are apparently produced during infection, suggesting that some physical difference between the two proteins allows SodCI, but not SodCII, to effectively combat phagocytic superoxide (Krishnakumar *et al.*, 2004). From our work and that of others, we know that SodCI is dimeric and protease resistant (Krishnakumar *et al.*, 2007;Ammendola *et al.*, 2008;Pesce *et al.*, 2000). We found that SodC from *Brucella abortus*, expressed from the *sodCI* locus, was able to complement *sodCI*, resulting in full virulence (Krishnakumar *et al.*, 2007). This enzyme is monomeric and released by osmotic shock but is protease resistant, suggesting that the latter property is critical in the phagocyte (Krishnakumar *et al.*, 2004;Krishnakumar *et al.*, 2007). We have also discovered that SodCI is "tethered" within the periplasm by a non-covalent ionic interaction. In vitro, this is displayed as limited release by osmotic shock (Krishnakumar *et al.*, 2004;Krishnakumar *et al.*, 2007). In the phagosome, *Salmonella* is also subjected to antimicrobial peptides, which disrupt membranes, similar to an osmotic shock (Vaara *et al.*, 1981). Therefore, we believe that tethering might also contribute to the ability of SodCI to protect *Salmonella* from phagosomal killing.

Taking all these data into consideration, our current working model proposes that macrophages deliver a variety of antimicrobial substances to the *Salmonella*-containing phagosome. CAMPs at least transiently disrupt the outer membrane of the bacterium. Periplasmic proteins such as SodCII are released and/or phagocytic proteases gain access to the periplasm, and SodCII is degraded under these conditions. The presence of CAMPs and additional phagosomal conditions induce the PhoPQ regulon, which includes *sodCI*, leading to resistance to antimicrobial peptides (Bader *et al.*, 2005;Golubeva and Slauch, 2006). SodCI is tethered within the periplasm and is

inherently protease resistant. Therefore, it remains to detoxify the phagocytic superoxide. Here we provide evidence in support of this model.

Consistent with this model, Uzzau et al. (Uzzau *et al.*, 2002) and Ammendola et al. (Ammendola *et al.*, 2005) have noted that the steady-state level of SodCII protein is low in bacteria recovered from macrophages or animals, whereas SodCI levels are high. However, they have proposed that the low levels of SodCII are due to transcriptional downregulation in response to the host environment. Most recently, these investigators proposed that *sodCII* transcription was downregulated in response to the low Zn levels found in the macrophage phagosome, although no molecular mechanism was proposed(Ammendola *et al.*, 2008). The experiments performed in this study distinguish between these two interpretations of the available data.

3.2 Materials and Methods

Media and reagents

Bacteria were grown in Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter), with 15 g of agar per liter added for solid medium. The concentrations of the antibiotics used were as follows: ampicillin and kanamycin, 50 µg/ml; chloramphenicol, 20 µg/ml; and tetracycline, 25 µg/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Fisher, *N, N, N', N'*-tetrakis (2-Pyridylmethyl) ethylenediamine (TPEN) was purchased from CalBiochem, and polymyxin sulfate B was purchased from Alexis. The 34-amino-acid mature Cathelicidin-

related antimicrobial peptide (CRAMP) was synthesized by the Protein Facility Center at the University of Illinois at Urbana-Champaign.

Strain construction

Bacterial strains and plasmids used in this study are described in Table 1. All strains used in this study are isogenic derivatives of *Salmonella enterica* serovar Typhimurium strain 14028. Deletion of genes and concomitant insertion of antibiotic resistance cassettes were carried out using lambda Red-mediated recombination (Datsenko and Wanner, 2000;Ellermeier *et al.*, 2002). All constructs were confirmed by PCR analysis and transduced into an unmutagenized wild-type background, using phage P22 HT105/1 *int-201* (Ellermeier *et al.*, 2002;Maloy S R *et al.*, 1996). In some cases, the antibiotic resistance cassette was recombined out of the chromosome by FLP, produced from the temperature-sensitive plasmid pCP20 (Datsenko and Wanner, 2000). Transcriptional *lac* fusions were constructed by integrating fusion plasmids into FRT scar sites by FLP-mediated recombination as described previously (Ellermeier *et al.*, 2002).

A *phoA*-6xHis allele was constructed by including a sequence encoding a 6xHis tag in the 3' PCR primer (CGC GGA TCC TTA CTA GTG GTG GTG GTG GTG GTG TTT CAG CCC CAG AGC G) and amplifying the *phoA* gene from the *Escherichia coli* chromosome (with 5' primer TTG TAC ATG AAG CTT ATA AAG TGA AAC AAA). The resulting PCR product contained a HindIII site 5' of the *phoA* open reading frame (ORF), which was in frame with the 6xHis sequence, followed by a stop codon and a BamHI site on the 3' end. This fragment was cloned into pWKS30 (Wang and Kushner, 1991). The resulting plasmid was introduced into a *phoN* deletion strain.

The *sodCI* gene was amplified using primers (5' primer, CGA GGT AAC ATA TGA AAT ACA C; and 3' primer, TTG CTC GAG TTT CTC AAT GAC AC) that introduced an NdeI site at the start codon and an AvaI site at the 3' end, replacing the stop codon. This fragment was cloned into the pET21b plasmid, resulting in the *sodCI* ORF being in frame with 6xHis-encoding sequences at the 3' end. This plasmid was introduced into an otherwise *sod* null strain that produced the T7 polymerase under the control of LacI (JS921). Therefore, SodCI-6xHis was produced upon the addition of IPTG.

The chromosomal *sodCI*-6xHis allele was constructed by incorporating sequences encoding 6xHis into the 5' primer (TGG TGG CGG TGCACG TTT TGC CTG TGG TGT CAT TGA GAA ACA CCA CCA CCA CCA CCA CTA GTA AGT GTA GGC TGG AGC TGC TTC G) used to amplify (with 3' primer CTT TAG GGT CGA GCG CAT TTC GTG CCC CAT ATA TGA ATA TCC TCC) a Kan^r cassette from plasmid pKD3 (Datsenko and Wanner, 2000). This cassette was integrated downstream of the *sodCI* open reading frame via lambda Red-mediated recombination, such that the 6xHis-encoding sequence was in frame with *sodCI*. The associated deletion extends through the left attachment site of the Gifsy-2 phage, which prevents phage induction or amplification (Golubeva and Slauch, 2006).

Antibody production and Western blot analysis

A peptide corresponding to the N-terminal 20 amino acids of mature SodCII was synthesized and used to raise antibody in a rabbit by Proteintech Group. Anti-His monoclonal antibody was purchased from Abcam, anti-β-galactosidase antibody was purchased from Zymed, and anti-chloramphenicol acetyltransferase (anti-CAT) rabbit

polyclonal antibody was purchased from Sigma. Horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Zymed) or HRP-conjugated anti-mouse antibody (Abcam) was used as secondary antibody. Protein samples were separated in a 10% polyacrylamide gel containing sodium dodecyl sulfate (SDS). The proteins were transferred to Hybond-ECL membranes (Amersham). The appropriate region of the membrane was excised and exposed to the indicated primary and secondary antibodies. The Western blot procedure and the detection of HRP-conjugated antibodies with a chemiluminescence system were done according to the manufacturer's instructions (Amersham). Control experiments (not shown) with appropriate deletion mutants confirmed the specificity of the various antibodies and the identities of the bands in our Western analyses.

Immunodetection in bacteria recovered from infected macrophages

RAW 264.7 macrophage-like cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone) supplemented with 10% fetal bovine serum and 1% L-glutamine (BioWhittaker). Macrophages were seeded into six-well plates at 1 x 10^6 to 5 x 10^6 cells/well. The *sodCI*$^+$*-6xhis-lacZ*$^+$ and *sodCII*$^+$*-lacZ*$^+$ fusion strains were grown for 16 h in LB with aeration. Bacterial cells were washed with sterile phosphate-buffered saline (PBS). An aliquot was frozen at –20°C; this was considered the LB sample. An aliquot of remaining bacterial cells was opsonized in 50% mouse serum for 30 min at 37°C. Bacteria were then diluted in DMEM and used to infect macrophages at a multiplicity of infection of ~10. After 30 min of incubation, the wells were washed three times with PBS, and then DMEM containing 12.5 µg of

gentamicin/ml was added to the wells to kill extracellular bacteria. After 20 min of additional incubation, the wells were washed once with PBS. DMEM with 12.5 µg of gentamicin/ml was added to the wells, and this was designated time zero. After 16 h, the wells were washed three times with PBS and macrophages were lysed with 1% Triton X-100. The released bacteria were washed with sterile distilled water and suspended in PBS. The number of bacteria in each sample was determined by plating dilutions of the sample. The remaining samples were frozen at –20°C. Based on the number of viable cells recovered, equal numbers (~10^6) of recovered bacteria and bacteria from the original LB culture were boiled in loading buffer, and immunodetection was performed as described above.

Immunodetection in bacteria recovered from infected mouse spleens

The *sodCI*$^+$*-6xhis-lacZ*$^+$ and *sodCII*$^+$*-lacZ*$^+$ fusion strains were grown in LB with aeration for 16 h. An aliquot was frozen at –20°C (the LB sample). Cells were diluted in sterile 0.15 M NaCl to ~5,000 CFU/ml. An aliquot was diluted and plated to determine the actual number of cells. For each strain, two BALB/c mice were infected intraperitoneally with ~1,000 cells. Bacterial cells were extracted from splenic tissue as described previously (Slauch *et al.*, 1994). In short, after 4 days of infection, the two mice were sacrificed, and the spleens were recovered and homogenized together in PBS. The samples were centrifuged, and to release the bacteria, the pellets were suspended in sterile deionized water containing 40 units bovine DNase (Sigma). The bacteria were recovered by centrifugation and suspended in PBS. Serial dilutions of each suspension were plated on LB agar to determine the number of viable bacteria. The remaining samples were

frozen at –20°C. Based on the number of viable cells recovered, equal numbers (~10^6) of recovered bacteria and bacteria from the original LB culture were boiled in loading buffer, and immunodetection was performed as described above.

Enzyme assays

Cyanide-sensitive SOD activity was determined using the xanthine oxidase-cytochrome *c* method (McCord and Fridovich, 1969). Glucose-6-phosphate dehydrogenase was assayed as described previously (Imlay and Imlay, 1996). The total protein concentration of each extract was measured using a Coomassie blue dye-based assay from Pierce.

In most cases, SodC activity was determined in strains that are otherwise *sod* nulls and in which cyanide-resistant SOD activity, which we routinely measure, is negligible. To measure the effects of the chelators EDTA and TPEN on SodC activity, SodCI- or SodCII-expressing plasmids were introduced into a Δ*sodCI* Δ*sodCII* strain. This strain still produces cytoplasmic SodA and SodB. It is known that chelators can inhibit growth of *sodA sodB* mutants (Maringanti and Imlay, 1999). The strains were grown for 16 h in LB and the indicated amount of EDTA or TPEN with aeration. These strains grew well under all of these conditions. Cyanide-resistant SOD activity (SodA and SodB) was subtracted from total SOD activity to yield the activity contributed by SodCI or SodCII.

β-Galactosidase assays were performed using a microtiter plate assay as previously described (Slauch and Silhavy, 1991). In most cases, cultures were grown for 16 h in LB with aeration. For experiments examining the effects of EDTA and TPEN, cells were grown in 250 µl of LB in a 96-well plate while shaking at 37°C in an

ELX808IU microplate reader (Bio-Tek Instruments). This allowed us to easily monitor growth under these conditions. β-Galactosidase units are defined as (μmol of ONP formed min^{-1}) x 10^6/(OD_{600} x ml of cell suspension), where ONP is *ortho*-nitrophenol and OD_{600} is the optical density at 600 nm, and are reported as means ± standard deviations (n = 4).

Preparation of cellular fractions released by antimicrobial peptide or osmotic shock

Cells were grown in 20 ml of LB with aeration for 16 h. For whole-cell lysates, 10 ml of culture was centrifuged and cells were suspended in 1 ml PBS. The cells were disrupted in a French pressure cell, and the resulting lysate was centrifuged at 13,000 x *g* for 10 min at 4°C. The supernatants were used for Western analysis or to determine SodC activity. "Osmotic shockates" were obtained as described previously (Krishnakumar *et al.*, 2004). In short, 10 ml of the same cultures was centrifuged, and the cells were suspended in 5 ml 50 mM Tris, pH 7.0, 0.5 mM EDTA. Samples were centrifuged again, and cell pellets were suspended in 2 ml plasmolysis buffer (50 mM Tris, 2.5 mM EDTA, 20% [wt/vol] sucrose, pH 7.4). After sitting at room temperature for 10 min, the cells were centrifuged, suspended in 1 ml ice-cold double-distilled water (ddH_2O), and incubated on ice for 15 min. The cells were removed by centrifugation, and the supernatant was considered the osmotic shockate.

To obtain cationic antimicrobial peptide-released fractions, 20-ml aliquots of late-exponential-phase culture were washed and suspended in 1 ml of PBS. One sample was disrupted in a French pressure cell, and the resulting lysate was centrifuged at 13,000 x *g* for 10 min at 4°C. The supernatants were used for Western analysis or to

determine SodC activity (whole-cell extract; considered 100%). The remaining samples were treated with the indicated amount of polymyxin B or CRAMP antimicrobial peptide. After 20 min of incubation at 37°C, cells were spun down at 15,000 x *g* and supernatants were filtered (0.22-µm low-protein-binding filter; Millipore). The supernatants were used for Western analysis or to measure the amount of Sod activity released by antimicrobial peptide. In control experiments, the viability of cells before and after treatment was determined by plating dilutions of samples. Also, whole-cell extract and antimicrobial peptide-released fractions were assayed for glucose-6-phosphate dehydrogenase activity.

Mouse competition assays

For each competition assay, the two indicated strains were grown in LB for 16 h, mixed 1:1, and then diluted in sterile 0.15 M NaCl to 5,000 CFU/ml. Groups of 5 to 11 female BALB/c mice (Harlan Sprague Dawley, Inc.) or congenic *Cnlp*$^{-/-}$mice (CRAMP^{-} on a BALB/c background [(Pinheiro da *et al.*, 2009)]) were injected intraperitoneally (i.p.) with 0.2 ml of inoculum (approximately 1,000 total bacteria). Inocula were plated on LB and then replica plated onto the appropriate selective medium to determine the actual number and percentage of each strain used for the infection. The animals were sacrificed after 4 days of infection. The spleens were harvested and homogenized, and dilutions were plated on LB agar. Colonies were replica plated onto selective medium to determine the percentage of each strain recovered. A competitive index was calculated as follows: (percent strain A recovered/percent strain B recovered)/(percent strain A inoculated/percent strain B inoculated). Student's *t* test was used for statistical analysis. In most cases, the strains were rebuilt by phage P22-mediated transduction, and the mouse

virulence assay was repeated to confirm that the phenotype was the result of the designated mutation.

3.3 Results

SodCII is decreased posttranscriptionally in macrophages and mice

We hypothesized that SodCII is subject to proteolysis in the macrophage, accounting for decreased protein levels despite induced gene expression (Krishnakumar *et al.*, 2007). Other investigators have attributed low SodCII protein levels to transcriptional downregulation (Ammendola *et al.*, 2008;Figueroa-Bossi *et al.*, 2006). To explicitly distinguish these two models, we investigated SodCI and SodCII protein levels in bacteria recovered from RAW 264.7 macrophage cells or spleens of infected mice. We used transcriptional *lacZ* fusions in which the reporter gene was inserted immediately after the *sodCI* or *sodCII* stop codon (Golubeva and Slauch, 2006). Thus, we could simultaneously monitor the steady-state levels of SodC (I or II) and β-galactosidase by Western analysis. The strains also contained a cassette encoding CAT, which we monitored to ensure equal loading of gels. In the case of SodCII, the primary antibody used was raised against a peptide corresponding to the first 20 amino acids of the mature protein, and the construct encoded wild-type protein. The SodCI construct contained a C-terminal six-His tag, and protein levels were monitored using an anti-His-tag monoclonal antibody. The SodCI fusion construct was also associated with a deletion from the 3' end of the *sodCI* open reading frame through the left attachment site of the Gifsy-2 phage, thereby preventing phage induction or amplification, which could interfere with our

assays (Golubeva and Slauch, 2006). SodCI-6xHis retains full enzymatic activity, and strains encoding this *sodCI-6xhis* transcriptional *lacZ* fusion are fully virulent (data not shown).

These strains were used to infect both tissue culture macrophages and mice. Bacteria were recovered from tissue culture cells 16 h after infection. In the mouse infections, bacteria were recovered from spleens after 4 days, using a previously described procedure (Slauch *et al.*, 1994). In each case, the number of viable cells recovered was determined by plating for CFU. Control cultures were grown for 16 h in LB. Equal numbers of viable cells were loaded onto gels, and separated proteins were blotted to nitrocellulose. The molecular weights of β-galactosidase, CAT, and SodC (I or II) differ enough that the corresponding region of each blot could be cut out and probed for the respective protein. Figure.3.1. A and B show that the steady-state levels of both SodCI protein and β-galactosidase were significantly increased in bacteria recovered from macrophages and mice. This is consistent with previous data showing a transcriptional increase of *sodCI* upon infection and a corresponding increase in protein levels (Golubeva and Slauch, 2006;Uzzau *et al.*, 2002). The levels of β-galactosidase expressed from the *sodCII* fusion also increased, albeit to a lesser extent, consistent with previous data showing that *sodCII* is transcriptionally activated in the phagosome (Golubeva and Slauch, 2006). However, SodCII protein was barely detected in bacteria recovered from either macrophages or mice. The most plausible explanation is that SodCII is made but degraded in the phagosome, consistent with our model (Krishnakumar *et al.*, 2007). Degradation of SodCII could result from either bacterial proteases in the periplasm or phagosomal proteases. HtrA is the primary periplasmic protease that has previously been

implicated in *Salmonella* pathogenesis (Mo *et al.*, 2006). We tested possible SodCII degradation by HtrA during macrophage infection by repeating the above experiment in an *htrA* null background. SodCII was expressed in the *htrA* mutant background in LB, and the steady-state level of SodCII dramatically decreased in macrophages (Fig.3.1.C). This suggests that HtrA is not responsible for SodCII degradation during macrophage infection. We also know that SodCII, even without metal cofactors, is stable in the periplasm *in vitro* (Krishnakumar *et al.*, 2004).

The *sodCII* gene is not regulated in response to zinc

Campoy et al. (Campoy *et al.*, 2002) have shown that *Salmonella* mutants lacking ZnuA, the periplasmic Zn-binding protein component of the high-affinity Zn transport system, are attenuated, suggesting that the host environment is Zn limited. Ammendola et al. (Ammendola *et al.*, 2008) suggested that *sodCII* transcription is decreased in response to the low-Zn environment, although no specific mechanism was proposed. It is known that *sodCII* is regulated by the alternative sigma factor RpoS (Golubeva and Slauch, 2006;Fang *et al.*, 1999;Gort *et al.*, 1999); no other regulatory system is known to affect *sodCII* transcription in *Salmonella*. To clarify this issue, we asked if *sodCII* transcription was affected by the Zn concentration of the medium. We assayed β-galactosidase activity produced from the *sodCII-lacZ* fusion in both wild-type and *znuA* mutant backgrounds in the presence and absence of chelators. As controls for this experiment, we also monitored expression from single-copy *lacZ* fusions to *znuA*, known to be regulated in response to zinc (Patzer and Hantke, 1998), and *katE*, which is regulated by RpoS (Loewen and Hengge-Aronis, 1994) (Fig. 2A). Strains were grown in

LB, with or without 0.5 mM EDTA (the conditions used by Ammendola *et al*. [(Ammendola *et al.*, 2008)]) or 50 μM TPEN, a more specific zinc chelator (Shumaker *et al.*, 1998). The addition of either chelator caused a dramatic (>35-fold) increase in *znuA* expression, as expected (Fig. 2B). (Note that the *znuA* fusion creates a *znuA* null mutation.) The addition of EDTA or TPEN had little to no effect on expression of either *sodCII* or *katE* in a wild-type background. In the *znuA* mutant background, the presence of chelator did lead to a slight decrease in *sodCII* expression. Importantly, however, the *katE* fusion also decreased in expression under these conditions. It should be noted that the *znuA* mutant grows poorly at these concentrations of chelator. The simplest explanation for these results is that under these restricted growth conditions, the RpoS regulon is not fully induced in the *znuA* mutant.

The genes encoding the Znu transport system are repressed by Zur when the regulator binds Zn (Patzer and Hantke, 1998;Outten and O'Halloran, 2001). Figure 2C shows that in a *zur* null background, *znuA* expression was induced 40-fold, analogous to the induction seen in the presence of chelators. Induction of the high-affinity transport system in this mutant likely alters the intracellular concentration of Zn. However, expression of *sodCII* was unaffected by loss of Zur (Fig. 2C). These results indicate that Zur has no role in *sodCII* regulation. Together, these data strongly suggest that Zn has no direct role in *sodCII* expression.

Metal acquisition

SodC proteins acquire Cu and Zn adventitiously in the periplasm. The metal ions can also be removed from the cofactored proteins by chelation. It has been suggested (Ammendola

et al., 2008) that differential affinity and stability of metal binding in SodCI and SodCII contribute to their different roles in macrophage survival. We previously examined the steady-state levels of SodCI and SodCII recovered from bacteria grown in the presence of the Cu chelator TETA (Krishnakumar *et al.*, 2004). We performed a similar experiment examining the steady-state activities of SodCI and SodCII recovered from bacteria grown in the presence of the Zn chelator TPEN. We performed a parallel experiment examining the ability of the wild type versus the *znuA* mutant to grow in various concentrations of the chelator. In the absence of chelator, both SodCI and SodCII, expressed from *lac* promoters on equivalent plasmids, produced approximately equal absolute levels of SOD activity (Fig. 3A). Subsequent addition of Cu and Zn to the extracts showed that this represented 83% of recoverable SodCI activity and 30% of SodCII activity. At increasing concentrations of TPEN, the amounts of active SodCI and SodCII decreased, with SodCI being more active than SodCII at lower TPEN concentrations (Fig. 3A). At 20 µg/ml TPEN, both SodCI and SodCII activities were reduced to ~40% of those observed in the absence of chelator. The activities of both enzymes continued to decrease at higher concentrations. A similar trend was observed in our previous TETA experiments (Krishnakumar *et al.*, 2004). Interestingly, 20 µg/ml TPEN was also the concentration at which the *znuA* mutant started to show a growth defect (Fig. 3B). A *znuA* mutant was attenuated in an animal model (Campoy *et al.*, 2002); data not shown), suggesting that the phagosome might be limited in Zn. Under these conditions, it is possible that only a fraction of both SodCI and SodCII is active. However, their relative abilities to acquire metals do not seem to explain the different roles of these two enzymes in virulence.

Antimicrobial peptides cause release of periplasmic proteins

SodCI, unlike other periplasmic proteins, is not released by osmotic shock but rather is "tethered" within the periplasm by a non-covalent mechanism (Krishnakumar *et al.*, 2004;Krishnakumar *et al.*, 2007). One could argue that osmotic shock is not physiologically relevant. However, it is known that cationic antimicrobial peptides also cause the release of periplasmic proteins (Vaara *et al.*, 1981). We hypothesized that cationic antimicrobial peptides encountered during infection could cause the release of SodCII and other periplasmic proteins and/or expose the periplasmic proteins to phagocytic proteases. SodCI would remain tethered. To test this hypothesis, we compared the release of SodCI, SodCII, and *E. coli* PhoA, as a control, from *Salmonella* cells treated with sublethal concentrations of polymyxin B sulfate versus osmotic shock. In each case, proteins from untreated whole cells or in the supernatant from an equivalent number of treated cells were separated by SDS-PAGE, and the proteins of interest were detected by Western analysis. Figure 4 shows that both SodCII and PhoA were quantitatively released by osmotic shock. In contrast, virtually no SodCI was detectable in the supernatant of osmotically shocked cells, consistent with our previous data (Krishnakumar *et al.*, 2004;Krishnakumar *et al.*, 2007). When cells were treated with polymyxin B sulfate, significant percentages of SodCII and PhoA were released into the supernatant. Again, SodCI was not released under these conditions. Thus, both osmotic shock and treatment with polymyxin B sulfate cause the release of periplasmic proteins, but SodCI remains tethered within the periplasm.

The effect of polymyxin on SodC release from the periplasm was tested at various concentrations. Figure 5A shows that a maximum of 20% of SodCI was released, even in

the presence of 100 μM polymyxin. In contrast, almost 100% of SodCII was released under these conditions. We had previously constructed a monomeric SodCI allele by altering two amino acids in the dimer interface (Krishnakumar *et al.*, 2007). This enzymatically active mutant also happened to be released by osmotic shock (Krishnakumar *et al.*, 2007). As expected, this monomeric SodCI was also released by polymyxin treatment, to approximately the same extent as SodCII. It is important that the concentrations tested were sublethal concentrations of polymyxin. At 50 μM, no detectable amount of the cytoplasmic protein glucose-6-phosphate dehydrogenase was released into the supernatant (data not shown), and plating pelleted cells after treatment recovered 85% ± 11% of the starting CFU.

Mouse macrophages produce the cathelicidin-related antimicrobial peptide CRAMP, which is known to contribute to protection against *Salmonella* infection (Rosenberger *et al.*, 2004). We evaluated the effect of this antimicrobial peptide by using an *in vitro*-synthesized 34-amino-acid peptide corresponding to mature CRAMP (Gallo *et al.*, 1997). Upon treatment of *Salmonella* with increasing concentrations of CRAMP, SodCII was preferentially released compared to SodCI. Monomeric SodCI was also significantly released under these conditions (Fig. 5B). As described above, glucose-6-phosphate dehydrogenase was not released under these conditions (data not shown). Together, these data show that treatment with antimicrobial peptides leads to release of periplasmic proteins. SodCI is unusual in that it remains largely tethered within the periplasm.

SodCII can contribute to virulence in a constitutive PmrA background

Our model posits that SodCII is degraded by phagocytic proteases that gain access to the enzyme upon disruption of the outer membrane by CAMPs. If this is true, then SodCII should contribute to protection from phagocytic superoxide if it can be protected from proteolysis. The PhoPQ regulon is induced in response to the phagocytic environment. PhoPQ activation leads to induction of the PmrAB two-component system, which in turn induces expression of several genes whose products modify the structure of lipopolysaccharide (LPS), conferring increased resistance to CAMPs (Gunn, 2008). Mutations that constitutively activate the PmrA sensor kinase confer resistance to polymyxin and other CAMPs (Vaara *et al.*, 1981;Gunn, 2008;Roland *et al.*, 1993). We compared the release of SodCII upon treatment with polymyxin or CRAMP in *pmrA* (Con) versus wild-type backgrounds. As described above, a large amount of SodCII was released from the wild-type cells by treatment with either 100 μM polymyxin or 50 μM CRAMP. In contrast, little SodCII was released from the *pmrA*(Con) bacteria under the same conditions (Fig. 6A). The *pmrA*(Con) mutation had no effect on the regulation of *sodCII*, whereas the known PmrAB-regulated gene *pmrI* (Tamayo *et al.*, 2005) was clearly induced in this background (Fig. 6B).

We then tested if SodCII can contribute to virulence in a mouse model of infection when protected from antimicrobial peptides in a *pmrA*(Con) background. First, we competed a *sodCI* mutant (which produces wild-type SodCII) against a *sodCI sodCII* double mutant. In an i.p. competition assay, these two strains competed evenly (Table 2). This finding is identical to previous results (Krishnakumar *et al.*, 2004;Uzzau *et al.*, 2002) and shows that in a wild-type background, SodCII does not contribute to virulence, even in the

absence of SodCI. We then performed the same competition assay, except that both strains also contained the *pmrA*(Con) mutation. In this background, the strain that produced SodCII outcompeted the strain lacking periplasmic superoxide dismutase. This result suggests that when *Salmonella* is protected from antimicrobial peptides, SodCII can function and contribute to virulence, consistent with our model.

SodCII can contribute to virulence in *Cnlp*$^{-/-}$ knockout mice

Next, we tested if SodCII can contribute to virulence in a *Cnlp*$^{-/-}$mouse, which cannot produce CRAMP (Pinheiro da *et al.*, 2009). First, we competed a *sodCI* mutant against a wild-type strain in *Cnlp*$^{-/-}$ mice to ensure that these knockout mice produce phagocytic superoxide (Craig and Slauch, 2009). The data in Table 2 show that the *sodCI* mutant was attenuated 10-fold compared to the wild-type strain, similar to the phenotype conferred in normal BALB/c mice (Krishnakumar *et al.*, 2004). Second, the *sodCI sodCII* double mutant was competed against the *sodCI* single mutant. The strain lacking SodCII was attenuated compared to the *sodCII*$^{+}$ strain. Indeed, the competitive index was strikingly similar to the results observed in the *pmrA* (Con) background. Thus, either infection of mice that cannot produce CRAMP or protection of the outer membrane by constitutive induction of the PmrAB regulon establishes conditions where SodCII is capable of protecting against phagocytic superoxide.

3.4 Discussions

Salmonella serovar Typhimurium strain 14028 produces two periplasmic superoxide dismutases, SodCI and SodCII. Although some original data suggested that mutations in *sodCII* attenuated virulence, there is now consensus that only SodCI contributes to protection of *Salmonella* from phagocytic superoxide (Krishnakumar *et al.*, 2004;Krishnakumar *et al.*, 2007;Ammendola *et al.*, 2008;Uzzau *et al.*, 2002); SodCII plays no role during infection, even in stains lacking SodCI. Presumably, SodCII does protect against periplasmic superoxide in some environment or condition outside the host (Korshunov and Imlay, 2002). Why do virulent strains require the additional phage-encoded enzyme SodCI to survive in macrophages? What prevents SodCII from functioning in the phagosome? The inability of SodCII to contribute to virulence is likely due to multiple factors. SodCI has a slightly higher specific activity (Ammendola *et al.*, 2008). The two enzymes might also differ in the ability to function under metal-limiting conditions (Ammendola *et al.*, 2008),although they respond similarly when cells are grown in the presence of either Cu or Zn chelators (Krishnakumar *et al.*, 2004) (Fig. 3). Yet these subtle differences in enzymatic activity do not seem sufficient to explain the complete inability of SodCII to contribute to protection against phagocytic superoxide. The genes encoding SodCI and SodCII are also differentially regulated. However, this differential regulation also does not explain the disparity in function (Krishnakumar *et al.*, 2004). The *sodCI* gene is controlled by PhoPQ and is induced ~17-fold in macrophages (Golubeva and Slauch, 2006). In this study, we confirmed the *sodCI* transcriptional induction and a corresponding increase in the amount of protein, consistent with recent

reports (Uzzau *et al.*, 2002;Ammendola *et al.*, 2008). Expression of *sodCII* is controlled by RpoS (Golubeva and Slauch, 2006). There is no evidence of additional regulation. Our data show that this gene is not controlled in response to Zn levels, as recently suggested (Ammendola *et al.*, 2008). We have previously shown that *sodCII* is induced 3- to 4-fold in macrophages and that induction is RpoS dependent (Golubeva and Slauch, 2006). In this study, we confirmed this transcriptional induction in macrophages and mice. However, the steady-state level of SodCII protein did not increase proportionally (Fig. 1). This is consistent with previous reports showing low levels of SodCII protein during infection (Ammendola *et al.*, 2008;Uzzau *et al.*, 2002), although it should be noted that the Flag-tagged SodCI and SodCII proteins used by these investigators are enzymatically inactive (Figueroa-Bossi *et al.*, 2006); data not shown). The constructs used in our study are fully active.

Accounting for all the data, our current working model proposes that macrophages deliver a variety of antimicrobial molecules to the *Salmonella*-containing phagosome, including antimicrobial peptides, proteases, and superoxide. The antimicrobial peptides at least transiently disrupt the outer membrane of the bacterium. Periplasmic proteins such as SodCII are released and/or phagocytic proteases gain access to the periplasm. SodCII is degraded under these conditions. In contrast, SodCI is transcriptionally induced in the phagosome, is tethered within the periplasm, and is inherently protease resistant. These properties allow the protein to detoxify phagocytic superoxide in the face of these additional antimicrobial effectors.

Consistent with this model, we have shown here that if the bacterium is protected from the action of antimicrobial peptides, then SodCII can contribute to pathogenesis. SodCII

does not fully replace SodCI under these conditions. This finding suggests that protection from degradation may be incomplete. Indeed, we could not detect a significant increase in the steady-state level of SodCII in the *pmrA* (Con) background in either macrophages or mice (data not shown), but we know that additional factors are important for protection against antimicrobial peptides. This is evidenced by the fact that a *phoP* mutant is sensitive to a >100-fold lower concentration of CRAMP than that required to inhibit a *pmrA* mutant (data not shown). We could not use *phoQ* constitutive mutants in our experiments because these strains are highly attenuated (Miller and Mekalanos, 1990). As outlined above, it is also possible that other factors contribute to the robustness of SodCI action during growth in macrophages.

Our results imply that as *Salmonella* cells adapt to grow in the macrophage, the periplasmic contents are vulnerable to loss or degradation. Although not all periplasmic proteins would be equally susceptible, it is likely that other periplasmic proteins, in addition to SodCII, are damaged or lost. It depends on the function of the protein whether simple leakage would affect function. For example, we have shown that SodC from *Brucella abortus*, although released by osmotic shock, is protease resistant and can complement *sodCI* and protect against phagocytic superoxide(Krishnakumar *et al.*, 2007). But periplasmic binding proteins involved in transport, for example, would be nonfunctional if separated from their membrane components.

Despite this apparent vulnerability, *Salmonella* cells do adapt and propagate in the macrophage. This is likely a recurring battle, as most macrophages in the host have only a few *Salmonella* organisms, suggesting that bacteria are constantly being engulfed by new macrophages and must adapt to the new environment (Mastroeni *et al.*,

2009). Alternatively, once a bacterium adapts, it would be fully prepared for the new macrophage. If our model is correct, we would expect SodCII levels to recover and contribute to protection under these conditions. It is also known that superoxide acts early during macrophage infection, which could further reflect this window of vulnerability during adaptation (Vazquez-Torres *et al.*, 2000b).

Surprisingly little is known about the mechanism by which various antimicrobial effectors of macrophages damage or kill bacteria. Even less is known about the interactions between these killing mechanisms. Evidence suggests that serine proteases are required to proteolytically activate CRAMP (Rosenberger *et al.*, 2004) and that reactive oxygen-dependent signaling is required to activate transcription of the gene encoding CRAMP (Rosenberger *et al.*, 2004) as well as other defenses (Forman and Torres, 2002;Gwinn and Vallyathan, 2006). It is also known by *in vitro* work that antimicrobial peptides can act synergistically and can facilitate the action of lysozyme by overcoming the outer membrane barrier (Yan and Hancock, 2001), but in vivo data are lacking. Our work provides direct evidence that antimicrobial effectors of macrophages do not act independently; rather, they cooperate to damage bacteria. Namely, antimicrobial peptides allow proteases access to periplasmic proteins, including the bacterial defense against phagocytic superoxide. We have provided evidence that the primary target(s) of superoxide is extracytoplasmic (Craig and Slauch, 2009), but whether the ability of superoxide to inhibit or kill bacteria is directly enhanced by the action of other effector functions, such as antimicrobial peptides, is an unknown but interesting concept.

3.5 Tables and Figures

Table 3.1 Strain list

Name	Genotype [a]	Deletion endpoints [b]	Source or reference [c]
14028	Wild type		ATCC [d]
JSG435	*pmrA505 zjd*::Tn*10d*-Cam		(Gunn and Miller, 1996)
JSG1050	*pmrI*::MudJ		(Tamayo *et al.*, 2005)
TN3740	LT2 *leu-485 trp190*::(*lacI lacp T7pol spc*)		C.G. Miller
JS531	Φ(*sodCII*$^+$-*lac*$^+$)*110*	1516586-1516598	(Golubeva and Slauch, 2006)
JS900	Φ(*sodCI*$^+$-*6xhis-lac*$^+$)*2920*	1098178–1130040	
JS901	Δ*htrA*::Kan	244465-245933	
JS902	Δ*katE11*::Cam	1397114-1399406	
JS903	Δ*zur*::Cam	4461801-4462315	
JS904	Δ*oppA-F*::Cam	1858868-1841314	
JS905	Φ(*sodCI*$^+$-*6xhis-lac*$^+$)*2920 zyd*::Tn*10d*-Cam		
JS906	Φ(*sodCII*$^+$-*lac*$^+$)*110 zyd*::Tn*10d*-Cam		
JS907	Φ(*sodCII*$^+$-*lac*$^+$)*110* Δ*htrA*::Kan *zyd*::Tn*10d*-Cam		
JS908	Φ(*sodCII*$^+$-*lac*$^+$)*110* Δ*rpoS1191*::Tet		
JS909	Φ(*katE-lac*$^+$)*11*		
JS910	Φ(*katE-lac*$^+$)*11* Δ*rpoS1191*::Tet		
JS911	Φ(*znuA-lac*$^+$)		
JS912	Φ(*sodCII*$^+$-*lac*$^+$)*110* Δ*znuA*::Cam		
JS913	Φ(*katE-lac*$^+$)*11* Δ*znuA*::Cam		
JS914	Φ(*znuA-lac*$^+$) Δ*zur*::Cam		
JS915	Φ(*sodCII*$^+$-*lac*$^+$)*110* Δ*zur*::Cam		

Table 3.1 (Cont.)

JS916	Δ*sodCI -104*::Cam Δ*sodCII-103*::Kan /pMC101	
JS917	Δ*sodCI -104*::Cam Δ*sodCII-103*::Kan /pMC102	
JS918	Δ*znuA*::Cam	1986415-1987359
JS921	Δ*oppA-F*::Cam *trp190*::(*lacI lacp T7pol spc*) Δ*sodA101* Δ*sodB102* Δ*sodCI-104* Δ*sodCII-103 /*pBK103	
JS922	Δ*sodA101* Δ*sodB 102* Δ*sodCI::*Tet Δ*sodCII-105*::Cam /pMC102	
JS923	Δ*phoN*::Kan /pBK104	
JS924	Δ*sodA101* Δ*sodB102* Δ*sodCI*::Tet Δ*sodCII-105*::Cam /pMC101	
JS925	Δ*sodA101* Δ*sodB 102* Δ*sodCI*::Tet Δ*sodCII-105*::Cam /pRK106	
JS926	*zjd*::Tn*10d*-Cam /pMC101	
JS927	*pmrA505 zjd*::Tn*10d*-Cam /pMC101	
JS928	*pmrI*::MudJ	
JS929	*pmrI*::MudJ *pmrA505 zjd*::Tn*10d*-Cam	
JS930	Φ(*sodCII*$^{+}$-*lac*$^{+}$)*110 pmrA505 zjd*::Tn*10d*-Cam	
JS931	Δ*sodCI-104*::Kan *zjd*::Tn*10d*-Cam	
JS932	Δ*sodCI-104*::Kan *sodCII-106*::Tet *zjd*::Tn*10d*-Cam	
JS933	Δ*sodCI-104*::Kan *pmrA505 zjd*::Tn*10d*-Cam	
JS934	Δ*sodCI-104*::Kan Δ*sodCII-106*::Tet *pmrA505 zjd*::Tn*10d*-Cam	
JS935	Δ*sodCII-103*::Kan	
JS936	Δ*sodCI-104*::Cam Δ*sodCII-103*::Kan	

Table 3.1 (Cont.)

Plasmids		Cloned endpoints [b]	
pBK103	pET21b *sodCI-6xhis*	1130056-1130586	
pMC101	pWKS30 *sodCI*		(Krishnakumar *et al.*, 2004)
pMC102	pWSK29 *sodCII*		(Krishnakumar *et al.*, 2004)
pRK106	pWKS30 *sodCI* Y87E Y109E		(22)
pBK104	pWSK30 *phoA-6xhis*		
pKD46			(Datsenko and Wanner, 2000)
pCP20			(Datsenko and Wanner, 2000)
pKG136			(Ellermeier *et al.*, 2002)

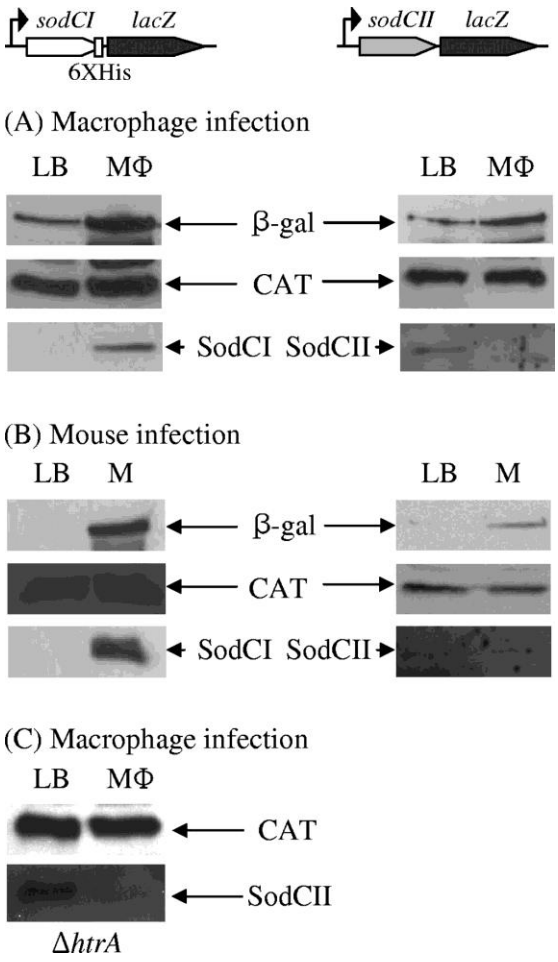


Figure 3.1 Expression and steady-state levels of SodCI and SodCII in vivo. Strains contain a chromosomal *lacZ* fusion in which the reporter gene is inserted just downstream of the stop codon for either *sodCI-6xhis* or *sodCII*. (A) Bacteria were recovered from LB after 16 h or from macrophages (MΦ) after 16 h of infection. (B) Bacteria were recovered from LB after 16 h or from mouse spleens (M) after 4 days of infection. (C) Same as in panel A, except that the background strain was a Δ *htrA* mutant. Whole-cell extracts were prepared from equal numbers of bacteria, separated by SDS-PAGE, and subjected to immunoblot analysis. Each set of panels represents a single gel for which the resulting nitrocellulose membrane was cut into sections and processed with primary antibody directed against the indicated protein. These results are representative of experiments performed 2 to 6 times. The strains used were JS905, JS906, and JS907.

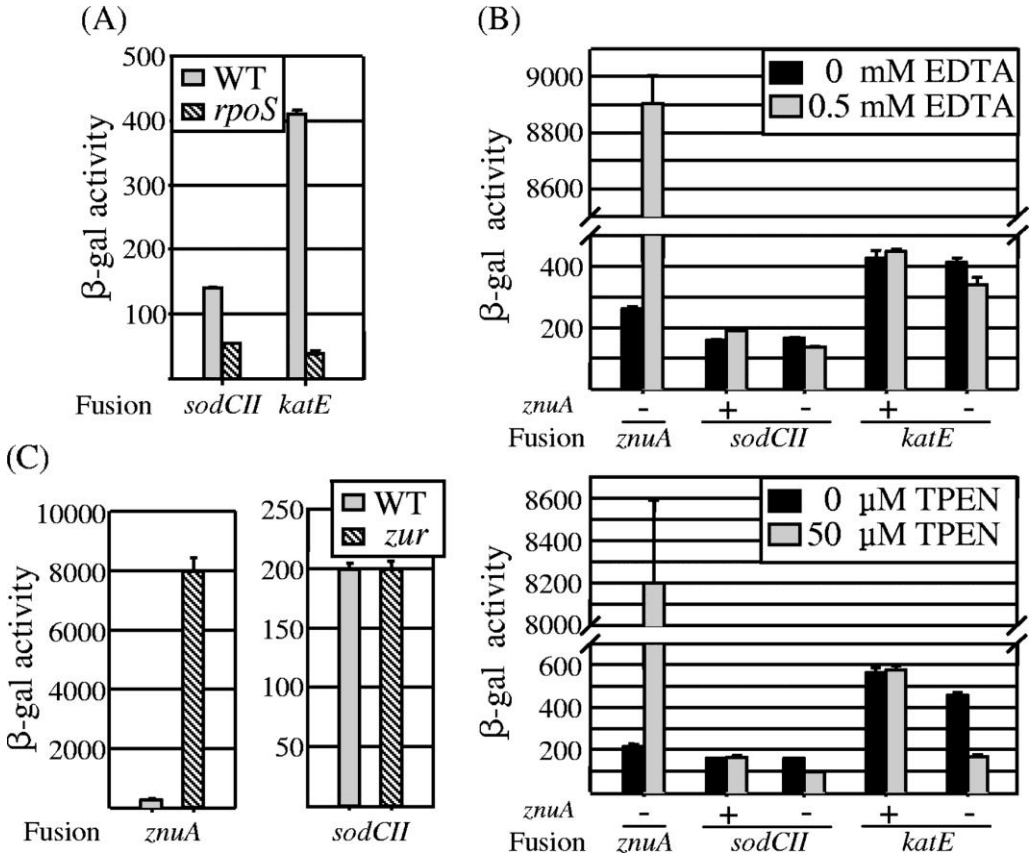


Figure 3.2 Transcription of *sodCII* is regulated by RpoS but is not directly affected by zinc level. (A) β-Galactosidase activity in strains containing *sodCII-lacZ* or *katE-lacZ* fusion in wild-type (WT) or *rpoS* null background, grown overnight in LB. The strains used were JS531, JS908, JS909, and JS910. (B) β-Galactosidase activity in strains containing *lacZ* fusions to the indicated genes in *znuA*$^+$ or *znuA* null background, grown overnight in LB containing either EDTA or the zinc chelator TPEN, as indicated. The strains used were JS911, JS531, JS912, JS909, and JS913. (C) β-Galactosidase activity in strains containing *znuA-lacZ* or *sodCII-lacZ* fusion in wild-type or *zur* null background, grown overnight in LB. The strains used were JS911, JS914, JS531, and JS915.

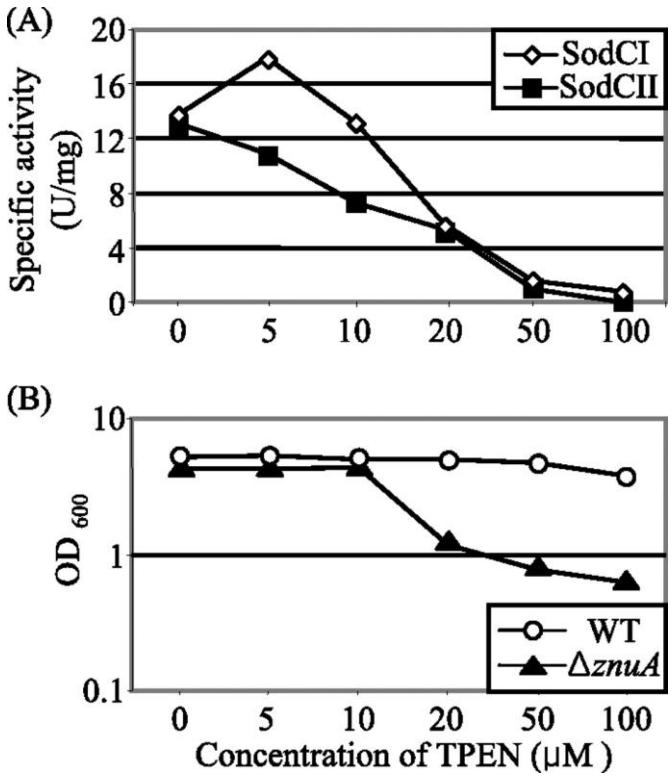


Figure 3.3 SodCI and SodCII activities in the presence of zinc chelator. (A) Strains producing SodCI or SodCII from a plasmid (in an otherwise *sod* null background) were grown overnight in LB with the indicated concentration of the zinc chelator TPEN. SOD-specific activity was determined from whole-cell extracts. (B) Wild-type and *znuA* null strains were grown overnight in LB with the indicated concentration of TPEN, and the resulting OD_{600} was determined. The strains used were JS916, JS917, 14028, and JS918.

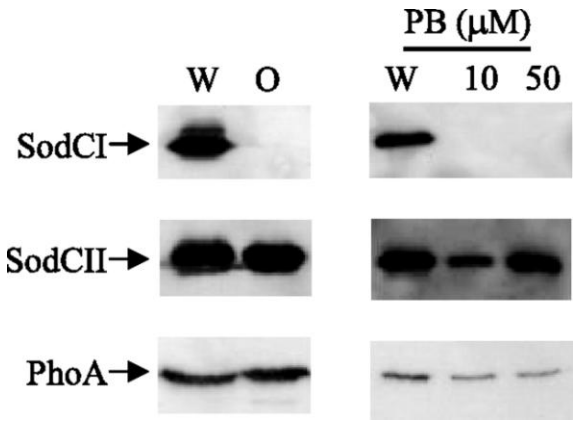


Figure 3.4 Polymyxin causes release of periplasmic proteins. The left panel shows the results of osmotic shock (O) as described in Materials and Methods. The right panel shows the results of treatment at the indicated concentration of polymyxin B (PB). In each case, a whole-cell extract (W) was compared to the supernatant from an equivalent number of cells after treatment. Proteins in each sample were separated by SDS-PAGE and subjected to immunoblot analysis with primary antibody directed against the indicated protein. The strains used were JS921, JS922, and JS923.

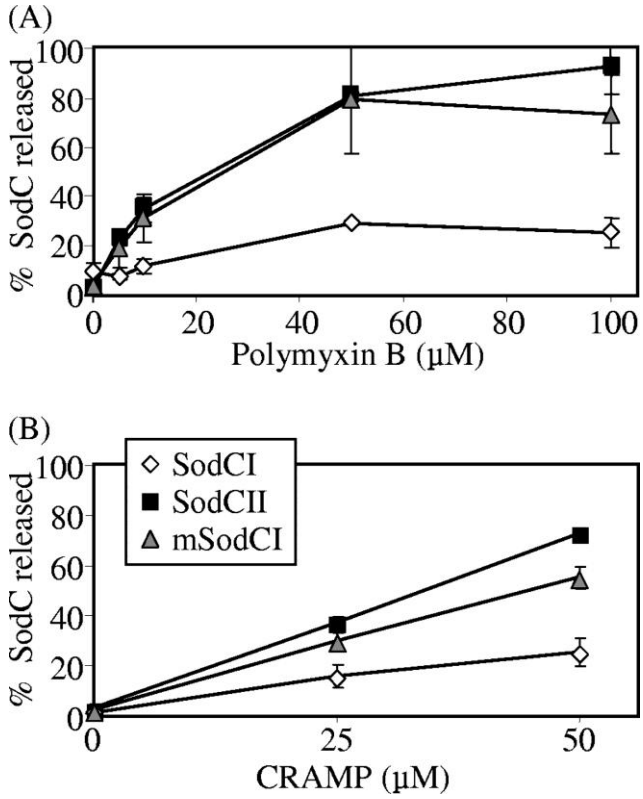


Figure 3.5 SodCII is preferentially released by polymyxin B and CRAMP. Strains producing SodCI, SodCII, or monomeric SodCI (mSodCI) from plasmids (in an otherwise *sod*-negative background) were treated with various concentrations of polymyxin B (A) or CRAMP (B). The SOD activity released into the supernatant was compared to that in a corresponding whole-cell extract. The strains used were JS924, JS922, and JS925.

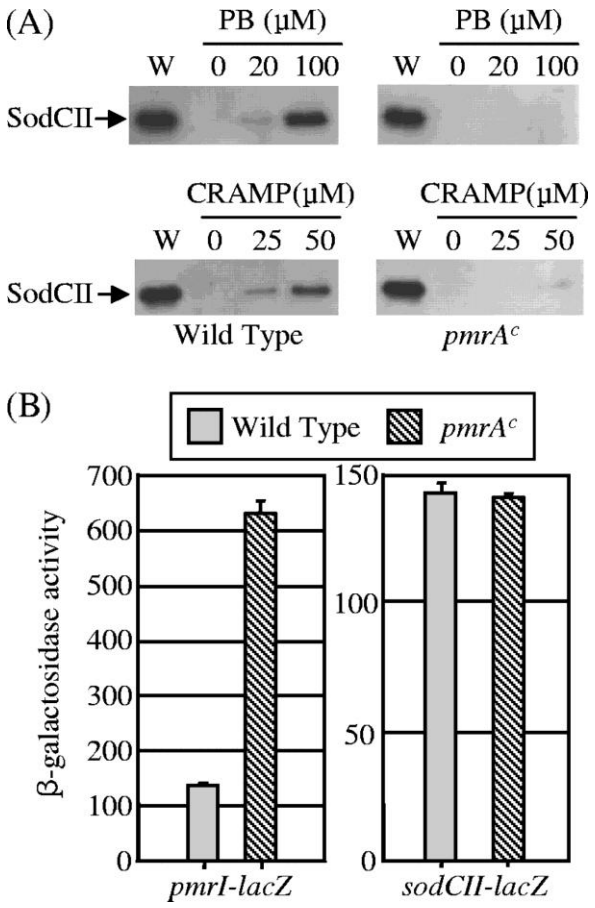


Figure 3.6 SodCII is not release by polymyxin B or CRAMP in a constitutive PmrA background. (A) Wild-type (left) and *pmrA*(Con) (right) strains were treated with the indicated concentration of polymyxin B (PB) or CRAMP. In each case, a whole-cell extract (W) was compared to the supernatant from an equivalent number of cells after treatment. Proteins in each sample were separated by SDS-PAGE and subjected to immunoblot analysis with anti-SodCII antibody. The strains used were JS926 and JS927. (B) β-Galactosidase activity in strains containing *pmrI-lacZ* or *sodCII-lacZ* fusion in wild-type or *pmrA*(Con) background, grown overnight in LB. PmrA does not control the transcription of *sodCII*. The strains used were JS928, JS929, JS906, and JS930.

Table 3.2

Mouse genotype	Salmonella Strain A	Salmonella Strain B	Median CI	No. of Mice	*P*
BALB/c	Δ*sodCI* Δ*sodCII*	Δ*sodCI*	1.14[§#]	14	NS
BALB/c	Δ*sodCI* Δ*sodCII pmrA*^{c}	Δ*sodCI pmrA*^{c}	0.58[§]	18	0.01
Cnlp-/- BALB/c	Δ*sodCI*	WT	0.01	2	0.002
Cnlp-/- BALB/c	Δ*sodCI* Δ*sodCII*	Δ*sodCI*	0.64[#]	11	0.013

Two experiments are significantly different (§*P*=0.046, # *P*=0.053)
NS : not significant

Chapter 4: *Salmonella* Periplasmic Stress Response During Infection in Mice

4.1 Introduction

Salmonella enterica serovar Typhimurium is an intracellular pathogen which survives and replicates in macrophages during the systemic infection of the host. *Salmonella* encounters both oxygen-dependent and -independent antimicrobial substances in the macrophage phagosomes. The oxygen dependent substances include superoxide and the independent ones are cationic antimicrobial peptides, proteases and lysozyme. These antimicrobial substances, including phagocytic superoxide, likely target extracytoplasmic components of the invading *Salmonella*. The *Salmonella* periplasmic superoxide dismutase, SodCI, is responsible for protection from phagocytic superoxide, but acts independently of cytoplasmic SOD (Craig and Slauch, 2009). This suggests that phagocytic superoxide affects an extracytoplasmic target(s) in *Salmonella*. Cathelicidin antimicrobial peptide (CRAMP) is known to be expressed in macrophages during *Salmonella* infection. CRAMP, like other cationic antimicrobial peptides, affects bacterial membrane integrity. At sublethal CRAMP concentrations, periplasmic proteins are released, while the cytoplasmic membrane apparently remains intact (Kim *et al.*, 2010). It has also been suggested that the inner membrane sensor, PhoQ, directly senses antimicrobial peptides via its periplasmic domain. Therefore it is hard to imagine that cationic antimicrobial peptides are not gaining access to the periplasmic space. *Salmonella* responds to envelope stress majorly via two overlapping stress response systems. The most extensively studied periplasmic stress response regulator is sigma E,

encoded by *rpoE*. *Salmonella rpoE* mutants are sensitive to antimicrobial peptides and reactive superoxide generating agents (Humphreys *et al.*, 1999;Testerman *et al.*, 2002). The virulence of an *rpoE* mutant is attenuated in wild type mice by either intraperitoneal or oral route (Humphreys *et al.*, 1999). The inability of the *rpoE* null mutant to cause disease after oral or intraperitoneal infection may be due to sensitivity to antimicrobial peptides and reactive oxygen species, but this has not been tested directly in vivo.

CpxRA is another major periplasmic stress response regulatory system. CpxRA comprise a two component system that regulates more than 100 genes, including the periplasmic protease encoding *htrA*, genes encoding periplasmic peptidylprolylisomerases *ppiA* and *ppiD*, and also *cpxRAP* (Pogliano *et al.*, 1997;Raivio and Silhavy, 1999;De *et al.*, 2002;Dorel *et al.*, 2006). Both a *cpxA* deletion strain and a strain containing the *cpxA** allele, which confers constitutive induction of the Cpx system, were attenuated during mouse infection. Interestingly, loss of the response regulator cpxR does not attenuate (Humphreys *et al.*, 2004). The reason this has not resolved.

The first gene identified as being regulated by sigma E was *htrA*, encoding the periplasmic protease also known as DegP. The *htrA* gene is also regulated by Cpx in *E. coli*, indicative of the partial redundancy of the two stress systems. The *htrA* mutant shows defective growth in hydrogen peroxide, but does not show increased sensitivity to polymyxin (Mutunga *et al.*, 2004;Humphreys *et al.*, 1999). An *htrA* deletion mutant cannot survive and replicate in wild type mice or macrophages in culture but is more virulent in gp97$^{-/-}$ mice, which do not produce superoxide.

Even though it has been known that these periplasmic stress response regulators are induced and important during infection, the host factors that induce these regulators has

not been elucidated in vivo. To answer this question and gain insight into the mechanisms by which phagocytes kill bacteria, we constructed RpoE- and Cpx-regulated *lacZ* fusions to measure the level of stress during infection in the mouse model and examined which antimicrobial components are responsible for the induction of each regulator.

4.2 Materials and Methods

Media and reagents

Bacteria were grown in Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter), with 15 g of agar per liter added for solid medium. The concentrations of the antibiotics used were as follows: ampicillin and kanamycin, 50 µg/ml; chloramphenicol, 20 µg/ml; and tetracycline, 25 µg/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Fisher, cecropin B was purchased from AnaSpec, and polymyxin sulfate B was purchased from Alexis. The 34-amino-acid mature cathelicidin-related antimicrobial peptide (CRAMP) was synthesized by the Protein Facility Center at the University of Illinois at Urbana-Champaign.

Strain construction

Bacterial strains and plasmids used in this study are described in Table 4.1. All strains used in this study are isogenic derivatives of *Salmonella enterica* serovar Typhimurium strain 14028. Deletion of genes and concomitant insertion of antibiotic resistance cassettes were carried out using lambda Red-mediated recombination (Datsenko and

Wanner, 2000;Ellermeier *et al.*, 2002;Ellermeier *et al.*, 2002). All constructs were confirmed by PCR analysis and transduced into an unmutagenized wild-type background, using phage P22 HT105/1 int-201 (Maloy S R *et al.*, 1996). In some cases, the antibiotic resistance cassette was recombined out of the chromosome by FLP, produced from the temperature-sensitive plasmid pCP20 (Datsenko and Wanner, 2000). To build the *htrA-lac* fusion, a CAT cassette was inserted immediately downstream of the *htrA* open reading frame such that the gene remained intact. The transcriptional lac fusion was constructed by integrating a fusion plasmid into the FRT scar site resulting from FLP-mediated recombination of the CAT cassette as described previously (Ellermeier *et al.*, 2002). The promoter of rpoE and cpxP fusion were cloned 5` to a promoterless *lacZ* gene in pDX1, an apramycin-resistant derivative of pAH125 (Haldimann and Wanner, 2001;Lin *et al.*, 2008). The resulting fusion plasmid was integrated into the *S. enterica* serovar Typhimurium chromosome at the lambda attachment site using Int produced from the CRIM helper plasmid pINT-ts (Haldimann and Wanner, 2001). The integrated plasmid was tested by PCR to ensure that only a single copy was present.

Growth conditions

For cationic antimicrobial sensitivity experiments, *Salmonella* were pre-grown in LB with aeration for ~1 hour then the indicated amount of polymyxin, polymyxin nonapeptide (PMNP), CRAMP or cecropin was added and the cultures were grown additionally for 2 hours. Resulting cultures were washed with saline and used to β-galctosidase assay. To see the effect of chemically produced superoxide, overnight xanthin and xanthine oxidase were used.

Immunodetection in bacteria recovered from infected mouse spleens

The *htrA*$^{+}$-*lacZ*$^{+}$ and *rpoE*$^{+}$-*lacZ*$^{+}$ fusion strains in the indicated mutant backgrounds were grown in LB with aeration for 16 h. Cells were diluted in sterile 0.15 M NaCl to ~ 500,000 CFU/ml. An aliquot was diluted and plated to determine the actual number of cells. For each strain, two BALB/c mice were infected intraperitoneally with ~100,000 cells. Bacterial cells were extracted from splenic tissue as described previously (Slauch *et al.*, 1994). In short, after 4 days of infection, the two mice were sacrificed, and the spleens were recovered and homogenized together in PBS. The samples were centrifuged, and to release the bacteria, the pellets were suspended in sterile deionized water containing 40 units bovine DNase (Sigma). The bacteria were recovered by centrifugation and suspended in PBS. Serial dilutions of each suspension were plated on LB agar to determine the number of viable bacteria. The remaining samples were frozen at –20°C. Based on the number of viable cells recovered, equal numbers (~10^{6}) of recovered bacteria and bacteria from the original LB culture were boiled in loading buffer, and immunodetection was performed as described below.

Antibody production and Western blot analysis

Anti-His monoclonal antibody was purchased from Abcam, anti-β-galactosidase antibody was purchased from Zymed, and anti-chloramphenicol acetyltransferase (anti-CAT) rabbit polyclonal antibody was purchased from Sigma. Horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Zymed) or HRP-conjugated anti-mouse antibody (Abcam) was used as secondary antibody. Protein samples were separated in a 10% polyacrylamide gel containing sodium dodecyl sulfate (SDS). The proteins were

transferred to Hybond-ECL membranes (Amersham). The appropriate region of the membrane was excised and exposed to the indicated primary and secondary antibodies. The Western blot procedure and the detection of HRP-conjugated antibodies with a chemiluminescence system were done according to the manufacturer's instructions (Amersham). Control experiments with appropriate deletion mutants confirmed the specificity of the various antibodies and the identities of the bands in our Western analyses.

4.3 Results

Salmonella htrA is mainly regulated by CpxR

In order to test if an envelop stress response is induced by antimicrobial peptides in *Salmonella*, a *lacZ* reporter fusion was introduced immediately after *htrA* open reading frame. This left *htrA* intact but allowed us to monitor transcription of the gene. We also constructed promoter *lacZ* fusions to *rpoE* and *cpxP*, which is reportedly the most highly regulated CpxR-dependent gene in the chromosome. In *E.coli*, it has been reported that *htrA* is regulated by both *rpoE* and *cpxR* (Danese *et al.*, 1995;Lipinska *et al.*, 1988;Pogliano *et al.*, 1997). We tested if this is also true in *Salmonella*. Expression of the htrA-lac fusion was reduced to approximately half that seen in the wild type strain. In contrast, there was significant *htrA* induction in the *rpoE* mutant background (Fig.4.1.A). Since, it is highly unlikely that *htrA* is reppressed by RpoE, we hypothesized that *htrA* is induced in *rpoE* background, not because of direct negative regulation, but because of indirect up regulation of Cpx regulon It is known that the Cpx and RpoE regulons can

partially compensate one another (Humphreys *et al.*, 2004). To test if there is compensatory induction in *Salmonella*, a *cpxP*- or *rpoE-lacZ* fusion was introduced into the *Salmonella* chromosome to monitor induction of the Cpx and RpoE regulons, respectively. Fig.4.1.B and C show that *cpxP* is induced in the *rpoE* deletion background, while *rpoE* is induced in a *cpxR* mutant. This suggests that *htrA* induction in the *rpoE* null is due to CpxR-dependent induction. Indeed, induction of htrA induction in the rpoE null is dependent on CpxR (Fig 4.1 A). These results show that htrA is primarily regulated by CpxRA in *Salmonella*. Therefore we used the *htrA* and *rpoE* lacZ fusions as representative reporters for the periplasmic stress response systems in *Salmonella*.

Expression of *htrA* is induced in a *pmrA* deletion strain during mouse infection

To test the effect of cationic antimicrobial peptides on *htrA* and *rpoE* expression during infection in mice, we monitored *htrA*- and *rpoE-lacZ* fusions in *pmrA* or wild type strains. Even though a *pmrA* mutation confers no direct phenotype during systemic infection by *Salmonella* (Gunn *et al.*, 2000), we have previously shown that a *pmrA* constitutive mutant can indirectly contribute *Salmonella* virulence in the macrophage (Kim *et al.*, 2010). The *htrA* or *rpoE* fusion strains were used to infect mice and bacteria were recovered from spleens after 4-5 days of infection. Proteins from recovered bacteria were separated by SDS PAGE and LacZ expression was monitored by immunoblot. Fig.4.2 shows that *htrA* expression is induced in *pmrA* mutant background compared with that of wild type. Expression of *htrA* in the $pmrA^c$ background was equivalent to that in the wild type strain. This is likely due to induction of of the PhoPQ and PmrAB regulons during infection, so it is difficult to distinguish between wild type and $pmrA^c$. In contrast, *rpoE*

expression was not induced in a similar experiment. These results suggest that *htrA* is induced in macrophages due to CAMP-mediated stress independent of *rpoE*.

Both *htrA* and *rpoE* are induced by polymyxin *in vitro*

To test the effect of cationic antimicrobial peptides on periplasmic stress response regulation in vitro, *htrA* and *rpoE* expression was monitored after growth in various concentrations of polymyxin. Both *htrA* and rpoE expression was induced at 1 μg/ml polymyxin (Fig.4.3.A). Next, we determined if *htrA* induction by polymyxin was dependent on CpxR or RpoE. Fig.4.3.A shows that *htrA* is not induced in the cpxR mutant background. This suggests that polymyxin induction of *htrA* is CpxR dependent. On the other hand, *htrA* was already fully induced in the *rpoE* null background, presumably because CpxR is induced to compensate for loss of RpoE. No further induction of *htrA* by polymyxin was detected in this background. Since an *rpoE* mutant is more sensitive to antimicrobial peptides, we could not test *cpxP* expression in polymyxin concentrations higher than 1 μg/ml. Therefore it is hard to determine that polymyxin dependent htrA induction is regulated by RpoE even though *rpoE* is evidently induced by polymyxin (Fig.4.3.C).

A higher concentration of antimicrobial peptide is needed to induce *htrA* in a *pmrA* constitutive background

To confirm that *htrA* and *rpoE* induction is polymyxin dependent, we introduced the *pmrA* constitutive mutant (*pmrA*^{c}) into *Salmonella*. The *pmrA*^{c} allele protects *Salmonella* from various cationic antimicrobial peptides, including polymyxin, by constitutively expressing enzymes responsible for adding amino-4-aminoarabinose to LPS, making

Salmonella LPS positively charged (Gunn *et al.*, 1998). The induction of *htrA* and *rpoE* in wild type and *pmrA*^{c} backgrounds was tested after growth in various concentrations of polymyxin. As expected, it required a higher concentration of polymyxin to induce *htrA* in the *pmrA*^{c} background. However, *htrA* induction was observed at 2ug/ml polymyxin (Fig.4.3.C).

The periplasmic stress response differs depending on the type of antimicrobial peptide

Since the *htrA* gene is induced in a *pmrA* null background during infection, we hypothesized that mouse macrophages produce certain types of antimicrobial peptides that up-regulate the Cpx system independent of *rpoE*. To test this hypothesis, we used three different cationic antimicrobial peptides. Cathelicidin related antimicrobial peptide (CRAMP) is known to be produced in in mouse macrophages (Rosenberger *et al.*, 2004). Cecropin is produced by insects and is thought to have a different mechanism of bacterial killing compared to polymyxin (Brogden, 2005). Polymyxin B nonapeptide (PMNP), which is a derivative of polymyxin, is able to release periplasmic proteins very effectively, but requires a 200 fold higher concentration to kill *E. coli* compared to polymyxin (Dixon and Chopra, 1986). We tested the possibility that these different kinds of cationic antimicrobial peptides can selectively induce either RpoE or HtrA. When cells were grown with CRAMP or PMNP, both *htrA* and *rpoE* expression were induced (Fig.4.4 A and B). This is similar to what was observed when cells were treated with polymyxin. In contrast, cecropin caused induction of *htrA* not *rpoE* expression. These data are consistent with previous reports suggesting different modes of action for the various antimicrobial peptides (Brogden, 2005). Apparently, these different mechanisms

of cell damage are differentially sensed by the periplasmic stress systems. Whereas CRAMP induces both systems in vitro, in vivo, only Cpx is induced, suggesting either that CRAMP might not be the predominant antimicrobial peptide encountered by *Salmonella*, or that other antimicrobial effectors in the phagosome alter the activity of CRAMP or response by the bacterium.

The RpoE regulon is induced in *sodC* mutants in mice

Craig and Slauch reported that the target of *Salmonella* periplasmic superoxide is extracytoplasmic (Craig and Slauch, 2009). It is reasonable to think that if phagocytic superoxide is damaging something within the bacterial envelope, the envelope stress response might be induced by superoxide. *Salmonella* produces two periplasmic superoxide dismutases, SodCI and SodCII. Although only SodCI contributes to virulence during infection, we deleted both the *sodCI* and *sodCII* genes for these experiments. We tested if *htrA* and *rpoE* expression was affected by superoxide during mouse infection. Interestingly, *rpoE* expression was induced in the *sodCI sodCII* background relative to the $sodC^{+}$ strain. In contrast, *htrA* expression was apparently decreased in the *sodCI sodCII* mutant. These data suggest that periplasmic superoxide dismutases protect from superoxide mediated damage that is sensed by RpoE.

Hydrogen peroxide or chemically produced superoxide cannot induce periplasmic stress *in vitro*

Expression of *htrA* and *rpoE* was monitored when cells were grown with the superoxide generating agents, xanthine /xanthine oxidase or pyrogallol. There was no induction of *htrA* or *rpoE* observed in the *sodCI sodCII* mutant (Fig.4.6). This is not surprising.

We have never observed phenotype in response to xanthine /xanthine oxidase and we believe that the amount of superoxide generated under these conditions is orders of magnitude lower than that encountered in the phagosome (Craig and Slauch, 2009). It is not clear why β-galactosidase activity was decreased in the presence of pyrogallol, but there was no different between the wild type and *sodCI sodCII* mutant.

It has been known that *htrA* or *rpoE* mutants are more susceptible to hydrogen peroxide. We monitored if *rpoE* or *htrA* expression was induced by hydrogen peroxide in vitro. Neither *htrA* nor *rpoE* expression was induced even at 1 mM hydrogen peroxide despite the fact that *Salmonella* growth was inhibited. These data suggest that there is no direct periplasmic stress induced by hydrogen peroxide.

The RpoE regulon is induced by proteases during infection

To test the effect of proteases during infection, we deleted the gene encoding periplasmic ecotin. *E. coli* ecotin mutants are more sensitive to neutrophil elastase compared to wild type. We compared *htrA* and *rpoE* expression in wild type and the *eco* null background during mouse infection. RpoE was induced in the *eco* mutant background, but *htrA* was not induced. Indeed, a compensatory decrease in *htrA* expression was apparent. We are currently testing if these systems are induced in vitro by the addition of proteases to the extracellular milieu.

CpxP does not have a direct role in *htrA* induction by polymyxin

Issac *et al.* showed that CpxP, a member of the Cpx regulon, is degraded by HtrA when P-pilin is over-expressed in the absence of its periplasmic chaperone (Isaac *et al.*, 2005).

Since CpxP is a CpxA autophosphorylation inhibitor, CpxP degradation leads to up-regulation of Cpx system. Their model posits that CpxP interacts with misfolded periplasmic proteins and both CpxP and the bound protein are targeted for degradation by HtrA, thus leading to induction of the Cpx system. However, in *Salmonella*, Cpx is not induced in an *htrA* deletion strain when grown in LB, but the system does turn on more strongly in the *htrA* deletion mutant compared to the wild type when the cells are treated with polymyxin (Fig.4.10). This is seemingly inconsistent with the *E. coli* reports that CpxP is degraded by HtrA with misfolded proteins. We directly tested if CpxP is degraded by HtrA during CAMP-mediated stress. The levels of FLAG-tagged CpxP protein and *cpx* transcription were measured in an *htrA* mutant and wild type. Both *cpxP* transcription and CpxP proteins levels increased upon treatment with polymyxin. However, it does seem that CpxP was being slightly degraded in that protein levels did not correlate with the increase in transcription and this was true in both wild type and *htrA* mutant backgrounds. Thus, CAMP-mediated CpxP protein degradation is not exclusively dependent on HtrA. One thing we noticed is that dimeric CpxP is detected in wild type extracts even in SDS PAGE. We tested if CpxP dimer is detected in *htrA*, *degQ*, *htrAdegQ* double mutants. (DegQ is also a highly conserved periplasmic protease.) Loss of HtrA led to loss of the dimer. The *degQ* mutant showed reduced dimer. CpxP may need to be dimeric to bind and inhibit CpxA. HtrA and DegQ may have some role in CpxP dimerization. In order to test these hypotheses, further studies are required.

4.4 Discussions

Salmonella responds to periplasmic stress mainly through the sigma E and Cpx systems. This periplasmic protein quality control is important for bacteria to withstand stresses such as high temperature, various chemicals, high pH, etc. *Salmonella* survives and replicates in macrophage during infection. The macrophage phagosome is a harsh environment where bacteria are subjected to CAMP, reactive oxygen species, low pH, and proteases. It has been shown that these antimicrobial components work in concert with each other (Rosenberger *et al.*, 2004;Kim *et al.*, 2010).

Proteins involved with periplasmic stress are also up-regulated during infection (Eriksson *et al.*, 2003) and deletion of those proteins often attenuate *Salmonella* virulence (Humphreys *et al.*, 2004;Humphreys *et al.*, 1999;Rowley *et al.*, 2005). We used *htrA* and *rpoE* chromosomal *lac* fusions to monitor periplasmic stress in the phagosome.

We found that *Salmonella* and *E. coli* apparently regulate *htrA* differently. By using an *htrA-lac* fusion immediately downstream of the *htrA* open reading frame, we believe that we are accurately measuring *htrA* transcription. Under our conditions, *htrA* was primarily regulated by CpxRA and indeed was induced in an *rpoE* mutant due to compensatory Cpx induction (Fig. 4.1). However, *htrA* does seem to be regulated when RpoE is highly induced such as in an *hfq* mutant (Fig XXX). Sigma E and CpxR from *E. coli* and *Salmonella* are virtually identical, but the *htrA* promoter has diverged. Therefore, one can imagine that the dynamics of Sigma E and CpxR binding to the *htrA* promoter can be different in *Salmonella* and *E. coli*. It has already been reported that the structures of the *E. coli* and *Salmonella htrA* promoters differ (Lewis *et al.*,

2009;Pogliano *et al.*, 1997). However, we did show a discrepancy with the data from Lewis *et al*. They identified three promoters upstream of the *Salmonella htrA* gene. One of these promoters was up-regulated upon cold shock in an RpoE-dependent manner. On the other hand, HtrA protein levels were slightly reduced when bacteria were grown at 42℃ on plates (), but they did not show any evidence of *htrA* transcriptional down regulation. It seems that they evaluated their data to fit the *E. coli* paradigm. Further study will be required to fully understand *Salmonella htrA* regulation.

HtrA (Cpx) was up-regulated in a *pmrA* mutant during infection, whereas RpoE was generally down-regulated possibly as a result of the compensatory effect of Cpx and Sigma E. We presume that loss of Pmr primarily results in increased susceptibility to antimicrobial peptides and, thus, these peptides are causing damage that is sensed by Cpx and not my RpoE. One could speculate that *htrA* and/or *rpoE* are regulated directly by PhoPQ, since the PhoPQ regulon is activated in a *pmrA* mutant (Gunn and Miller, 1996). But in vitro, neither *htrA* nor *rpoE* are affected by null mutations or constitutive alleles of either *pmrAB* or *phoPQ* (Fig.4.9), so this possibility is ruled out.

Both *htrA* and *rpoE* are induced when cells were grown in vitro with polymyxin and CRAMP, but only *htrA* is induced in cecropin. One major difference between cecropin and CRAMP is the mechanism of pore formation. CRAMP is a paralogue human of LL-37, which disrupts membrane by forming a toroidal pore in which peptides aggregate and induce the lipid monolayers to bend continuously through the pore so that the water core is lined by both the inserted peptides and the lipid head groups(Henzler Wildman *et al.*, 2003). On the other hand, cecropin disrupts the membrane by orienting parallel to the surface of the lipid bilayer and forming an extensive layer or carpet (Gazit *et al.*, 1995).

However, this is only speculation and does not address how these potentially different modes of action differentially affect the two periplasmic stress systems. But our results do leave us with a paradox. CRAMP is the only antimicrobial peptide known to be produced in mouse macrophages. Yet, our results would suggest that either CRAMP is not the primary factor causing damage under these conditions, or that the overall antimicrobial effectors combine in such a way to alter the response to CRAMP.

In contrast to what observed in the *pmrA* mutant, only RpoE was induced in the *sodCI sodCII* double mutant during infection (Fig.4.5). This is consistent with our data suggesting that phagocytic superoxide damages something extracytoplasmic. Again, this response cannot be mimicked in vitro with the addition of either superoxide or hydrogen peroxide (Fig.4.7). Thus, either the intensity of the damage is more severe in vivo or the damage is mediated by a combination of factors that cannot be replicated in vitro.

Likewise, only RpoE was induced in the ecotin mutant and this induction was seen only during infection. This is the most speculative situation in that we are assuming that loss of this factor increases sensitivity to phagocytic proteases. Ecotin has been shown not to inhibit the following *E. coli* proteases: Do, Re, Mi, Fa, So, La, Ci, Pi, and proteases I, II, IV, V and VI (Palmer and St John, 1987;Chung *et al.*, 1983). Therefore, it seems again that it is the overall combination of factors in the phagosome that is leading to the particular responses that we observe. The relationship between phagosomal proteases and the RpoE response will be studied further.

In conclusion, *htrA* is mainly regulated by Cpx in *Salmonella* but Sigma E can apparently override the Cpx system in certain conditions. Our data suggest that Cpx is up regulated

by phagosomal cationic antimicrobial peptides while Sigma E is up regulated by superoxide and macrophage proteases.

4.5 Tables and Figures

Table 4.1 Strain list

Name	genotype	Deletion or endpoint	Source or reference
BK425	*htrA*::cmR	245921-245960	
BK450	*htrA-lac*		
BK506	*cpx-lac*	4270246-4270764	pDX1(KG724)
BK508	*rpoE-lac*	278001- 2780292	pDX1(KG730)
JS327	*ΔcpxR*::cmR		(Ellermeier and Slauch, 2004)
BK710	*ΔcpxR*::TetR		
BK498	*ΔrpoE*::cmR	2779427-2780008	KG661
BK712	*ΔrpoE*::tetR		
BK725	*ΔhtrA*::TetR	244493-245919	
JS542	*phoQ24*::cmR		
JS545	*ΔphoP*::cmR		
JS570	*Δhfq*::kanR		(Ellermeier and Slauch, 2008)
BK466	*htrA*-lac/*ΔcpxR*::cmR		
BK723	*htrA*-lac/*ΔrpoE*::TetR		
BK706	*htrA*-lac/*ΔcpxR*::cmR *ΔrpoE*::TetR		
BK510	*rpoE*-lac/*ΔcpxR*::cmR		
BK512	*cpx*-lac/*ΔrpoE*::cmR		
BK537	*htrA-lac*/*phoP*::cmR		
BK535	*htrA-lac*/*phoQ24*		
BK881	*rpoE-lac/phoP*::cmR		
BK883	*rpoE-lac*/*phoQ24*		
BK490	*htrA*-lac/ *zjd*::Tnd-cm		
BK521	*htrA*-lac/ *ΔpmrA621 zjd*::Tnd-cm		
BK464	*htrA*-lac/*pmrAc zjd*::Tnd-cm		
BK553	*rpoE*-lac/ *zjd*::Tnd-cm		

Table 4.1 (Cont.)

BK575	*rpoE*-lac/*ΔpmrA621* *zjd*::Tnd-cm	
BK624	*ΔsodCI104 ΔsodCII*::TetR *zjd*::Tnd-cm	
BK582	*ΔsodCI104ΔsodCII*::TetR *zjd*::Tnd-cm / *htrA*-lac	
BK555	*ΔsodCI104 ΔsodCII*::TetR *zjd*::Tnd-cm / *rpoE*-lac	
BK790	*Δeco*::TetR	2360052-2360550
BK875	*htrA*-lac/*Δeco*::TetR *zjd*::Tnd-cm	
BK877	*rpoE*-lac/*Δeco*::TetR *zjd*::Tnd-cm	
BK773	*htrA-lac* / *Δhfq*	
BK737	*rpoE-lac* / *Δhfq*	
BK735	*cpx-lac* /*Δ hfq*	

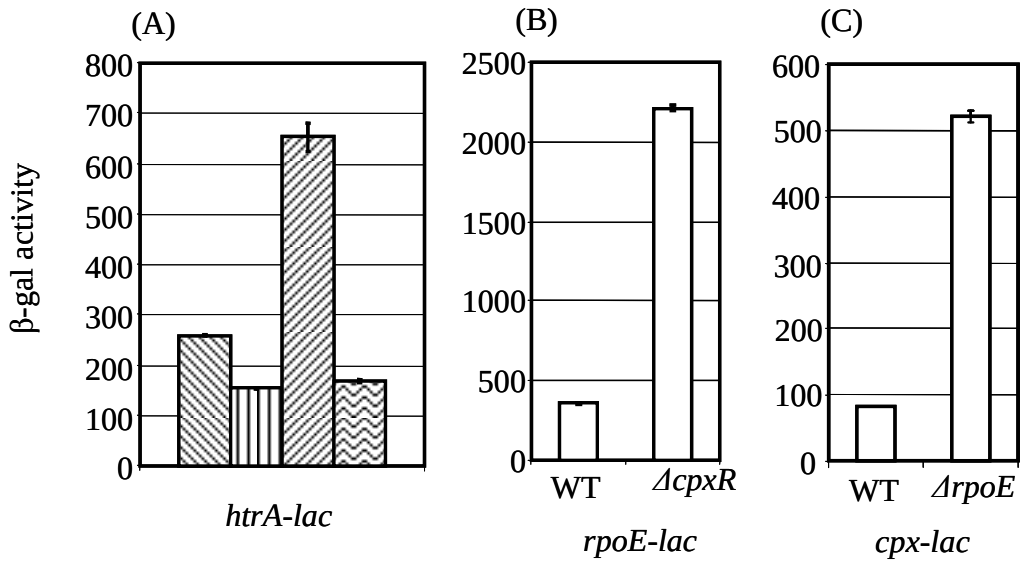


Figure 4.1 htrA is regulated by cpxR. (A) β-galactosidase activities in stain containing *htrA-lacZ* fusion in wild type (▨), *ΔcpxR* (▥), *ΔrpoE* (▨) and *ΔrpoEcpxR* (▨) mutant, grown overnight in LB. The strains used were BK450, BK466, BK 723, BK706 (B) β-galactosidase activities in stain containing *rpoE-lacZ* either in wild type or *ΔcpxR*, grown overnight. The strains used were BK508 and BK510. (C) β-galactosidase activities in stain containing *htrA-lacZ* either in wild type or *ΔrpoE*, grown overnight. The strains used were BK506 and BK512.

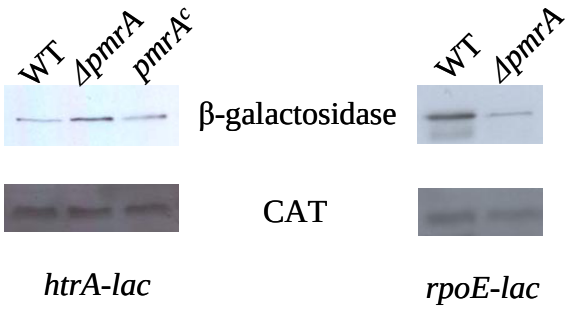


Figure 4.2 The effect of *pmrA* mutant on the expression of *htrA* and *rpoE* during mouse infection. Each *htrA*- or *rpoE*-lacZ fusion with indicating strain background strain was recovered from mouse spleen after 4 days of infection. Whole-cell extracts were prepared from equal numbers of bacteria, separated by SDS-PAGE, and subjected to immunoblot analysis. Each set of panels represents a single gel for which the resulting nitrocellulose membrane was cut into sections and processed with primary antibody directed against the indicated protein. These results are representative of experiments performed 3 times. The strains used were BK490, BK521, BK464, BK553 and BK575.

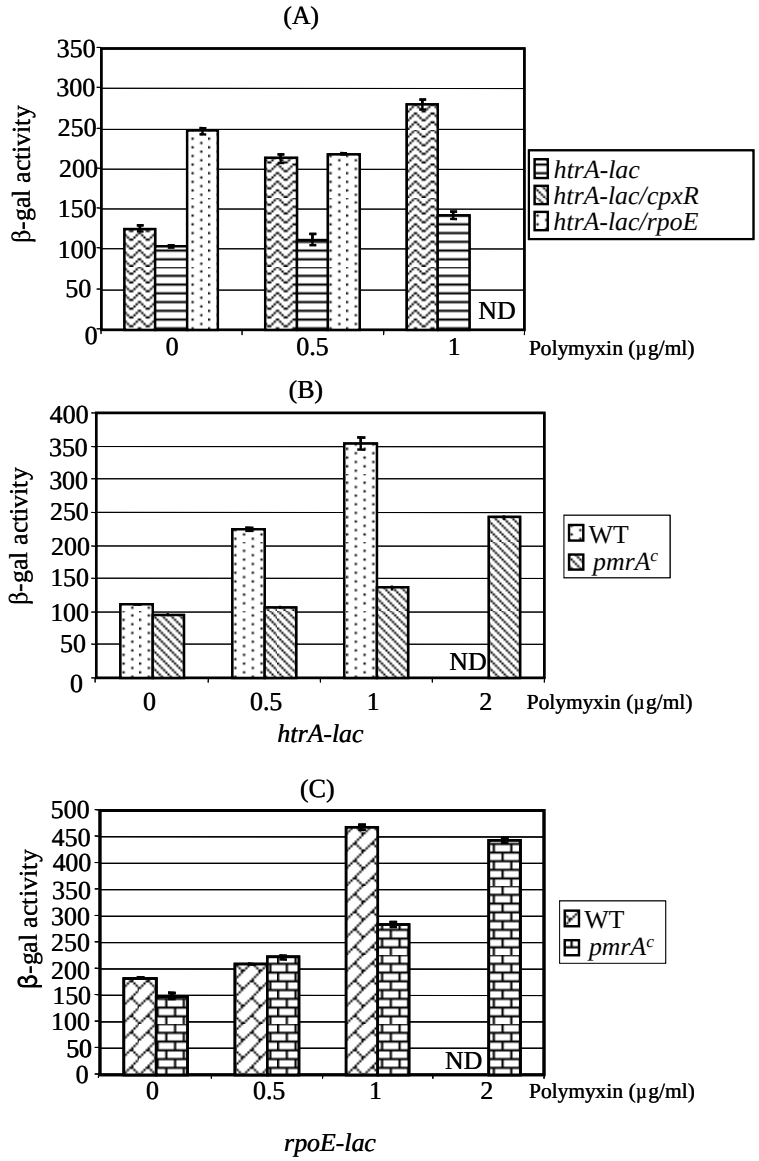


Figure 4.3 *htrA* and *rpoE* expression under polymyxin. β-galactosidase activity in strains grown with polymyxin. Cells which were pre grown in LB were grown with indicated amount of polymyxin. *htrA* expression was measured in either wild type, *ΔrpoE* and *ΔcpxR* background (A) or $pmrA^c$ background (B). *rpoE* expression is tested in wild type or $pmrA^c$ (C). ND : Not Done.

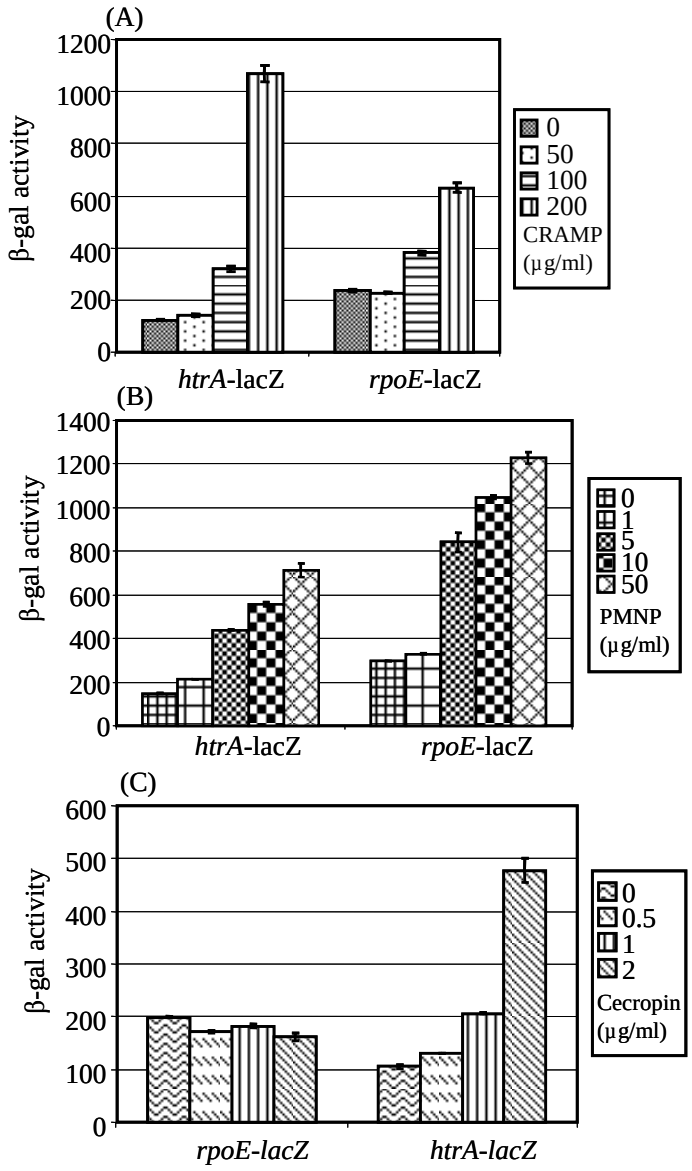


Figure 4.4 Effect of cationic antimicrobial peptide on htrA and rpoE expression. *htrA* or *rpoE-lac* fusion strain was grown in LB with indicated amount of Cathelicidin antimicrobial peptide (A), polymyxin nonapeptide (PMNP) and cecropin (C). The strains were used are BK 450 and BK 508.

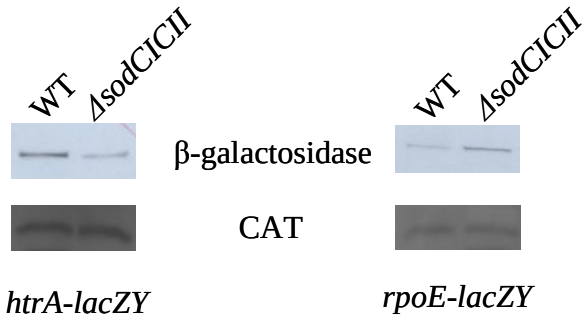


Figure 4.5 *rpoE* induction in the absence of SodCs during infection in mouse. Each bacterial strain with the either *htrA*- or *rpoE-lacZ* fusion with indicating strain background strain was recovered from mouse spleen after 4 days of infection. Whole-cell extracts were prepared from equal numbers of bacteria, separated by SDS-PAGE, and subjected to immunoblot analysis. Each set of panels represents a single gel for which the resulting nitrocellulose membrane was cut into sections and processed with primary antibody directed against the indicated protein. These results are representative of experiments performed 3 times. The strains used were BK490, BK553, BK582 and BK555.

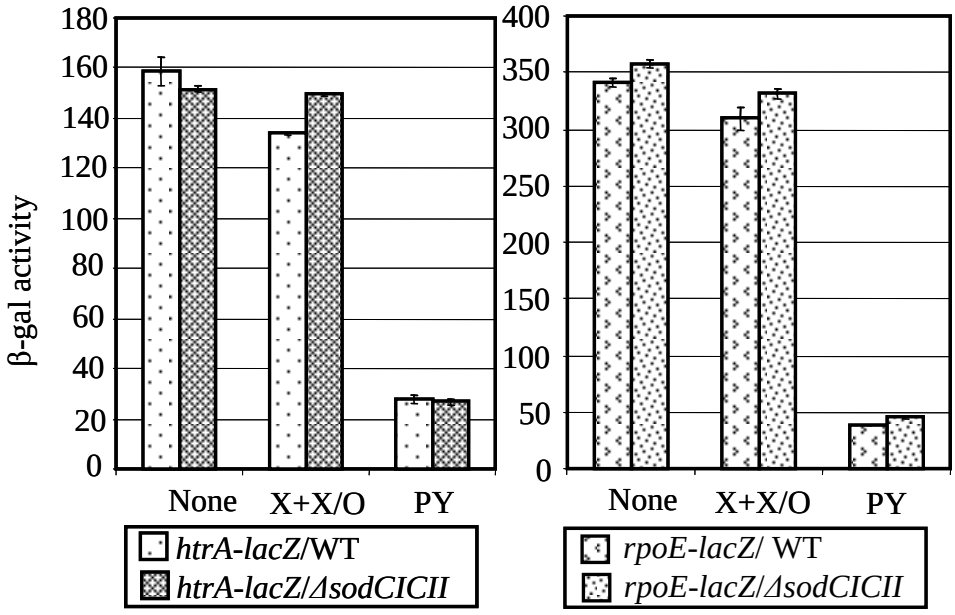


Figure 4.6 Chemically produced superoxide cannot induce either *htrA* or *rpoE* expression. Cells were grown with Xanthine and Xanthine oxidase (X and X/O) or pyrogallol (PY) and *htrA* or *rpoE* expressions were shown by β-galactosidase activity. The strains used were BK490, BK553, BK582 and BK555.

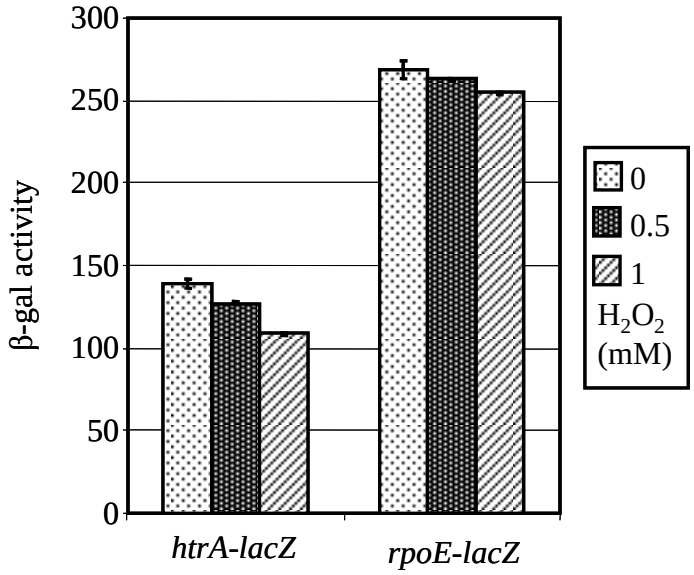


Figure 4.7 Hydrogen peroxide cannot induce either *htrA* or *rpoE* expression. *htrA* and *rpoE* lac fusion containing strains were grown with none or indicated amount of hydrogen peroxide. The strains used were BK490, BK553, BK582 and BK555.

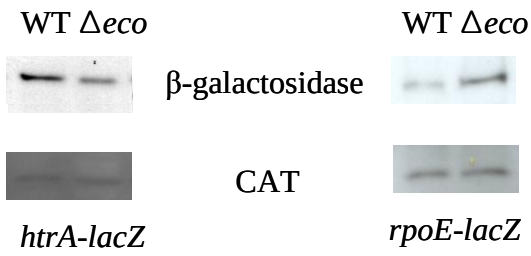


Figure 4.8 RpoE is induced in eco mutant during infection in mouse. The strains containing *htrA*- of *rpoE*- lac fusion with or without eco gene was used to infect mouse. The strains used were BK490, BK553, BK875 and BK877.

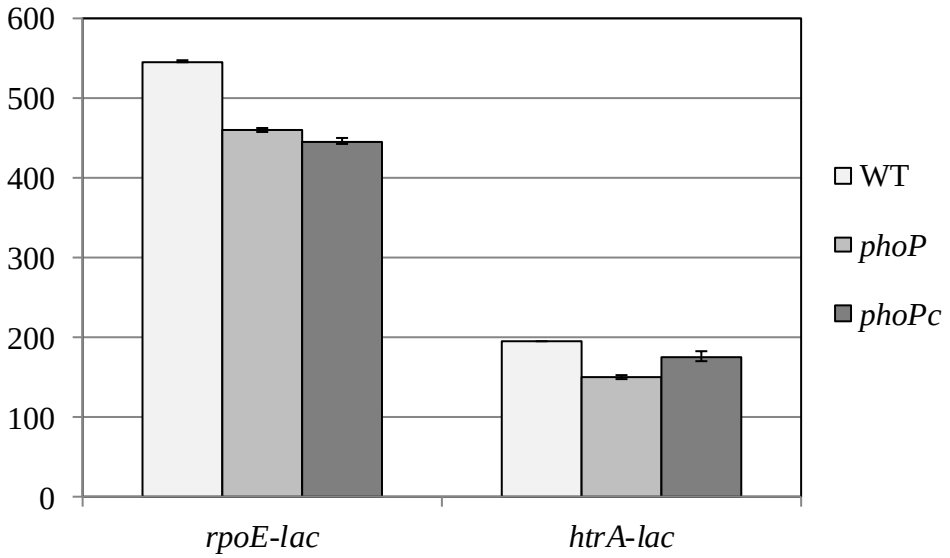


Figure 4.9 htrA and rpoE does not regulated by PhoPQ or PmrAB. β-galactosidase activity of *rpoE* and *htrA* lac fusion in wild type(WT), *phoP* deletion and *phoP* constitutive were measured. The strains used were BK450, BK508, BK535, BK 537, BK881 and BK883.

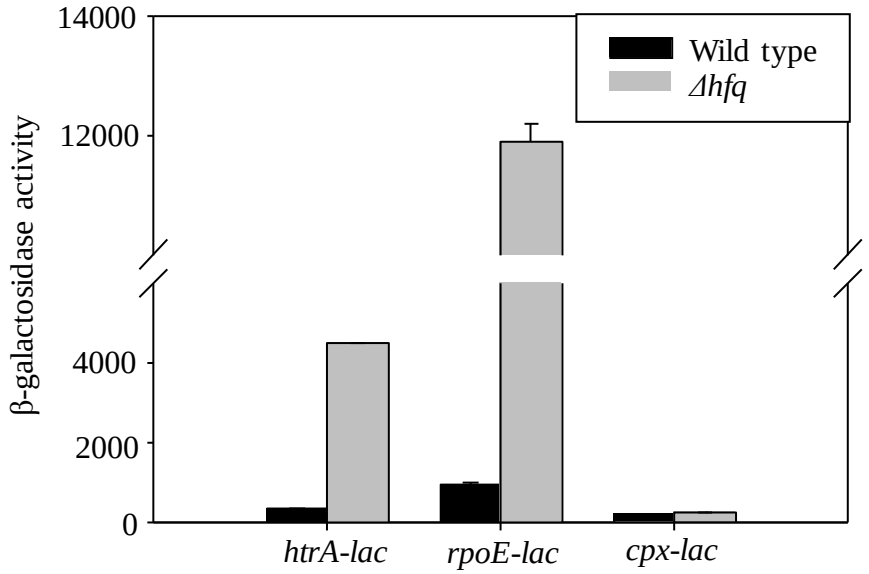


Figure 4.10 htrA regulated by RpoE in *hfq* mutant. β-galactosidase activity of *rpoE*, *htrA* and cpx lac fusion in wild type or hfq mutant were measured. Strains were grown overnight with aeration.The strains used were BK450, BK733, BK737, BK508, BK506 and BK753.

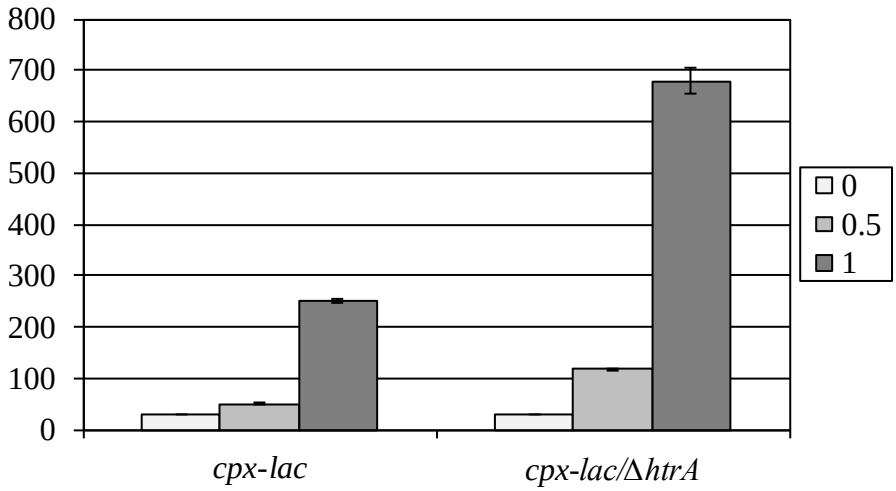


Figure 4.11 Loss of HtrA leads to increased *cpxP* induction upon treatment with polymyxin. Each overnight culture was subcultured 1:100 and grown for 1 hour. Polymyxin was added and the cultures were incubated for an additional hour. After growth, cells were washed and assayed for β-galactosidase activity. The strains used were BK679 and BK864The strains used were BK679 and BK864.

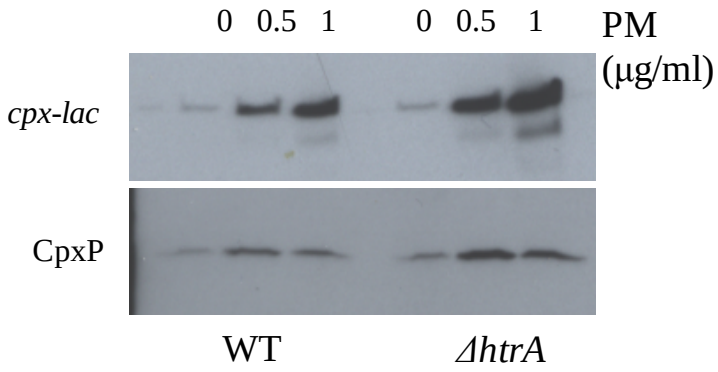


Figure 4.12 CpxP degrades in subleathal concentration of polymyxin. CpxP protein and cpx transcription level was measured by Immunoblot. Overnight cultures were inoculated 1:100 in LB and pre grown for one hour. Indicated amount of polymyxin was added and cells were grown an additional hour. The same number of cell were loaded based on OD600. Each set of panels represents a single gel for which the resulting nitrocellulose membrane was cut into sections and processed with primary antibody directed against the indicated protein. The used strains were BK679 and BK964.

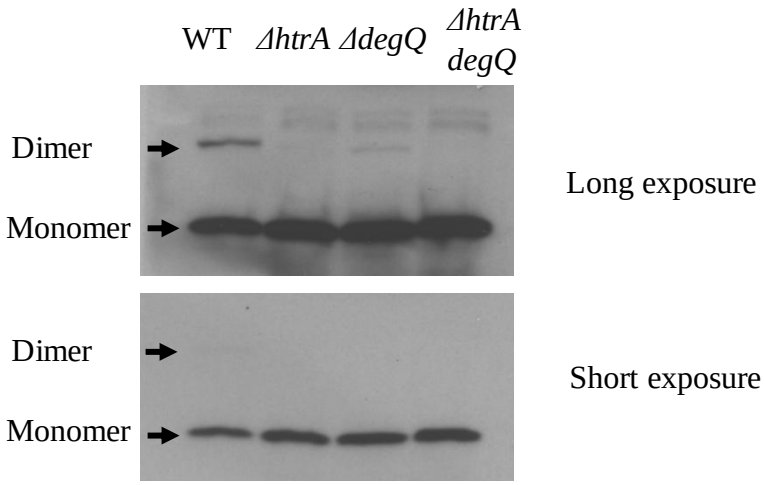


Figure 4.13 CpxP forms a dimer that is dependent on HtrA. Overnight cultures of indicated strain were used for SDS PAGE and Immunoblot. The two pictures are of the same blot but differ in exposure time. The strains used were BK679, BK826, BK840 and BK842.

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Curriculum Vitae

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PUBLICATIONS

Byoungkwan Kim, Susan M. Richards, John S. Gunn and James M. Slauch. Protecting SodCII in the phagosome allows it to contribute to virulence in *Salmonella enterica* serovar Typhimurium. ; *J Bacteriology.* 2010 Apr; 192(8):2140-9

Dongxia Lin*, **Byoungkwan Kim***, and James M. Slauch. DsbL and DsbI contribute to periplasmic disulfide bond formation in *Salmonella enterica* serovar Typhimurium. *Microbiology.* 2009 Dec; 155:4014-24 ***equally contributed author**

Radha Krishnakumar, **Byoungkwan Kim**, Elizabeth A. Mollo, James A. Imlay and James M. Slauch. Structural properties of periplasmic SodCI that correlate with virulence in *Salmonella enterica* serovar Typhimurium. *J Bacteriology.* 2007 Jun; 189(12):4343-52

Sangyong Lim, **Byoungkwan Kim**, Hyun-Sook Choi, Younghoon Lee and Sangryeol Ryu. Fis is required for proper regulation of *ssaG* expression in *Salmonella enterica* serovar Typhimurium. *Microb Pathog*. 2006 Jul; 41(1):33-42

CONFERENCES AND MEETING

Oral presentation

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